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GENETIC POLYMORPHISM IN DRUG OXIDATION: *IN VITRO* STUDIES OF HUMAN DEBRISOQUINE 4-HYDROXYLASE AND BUFURALOL 1′-HYDROXYLASE ACTIVITIES

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Abstract—1. A sensitive, specific assay utilizing fluorescence-HPLC has been developed for determining the 1′-hydroxylation of bufuralol by human liver.

2. The 1′-hydroxylation of the isomers of bufuralol varied threefold, both the V_{max} and the K_m for the (+) isomer being greater than the corresponding values for the (−) isomer.

3. Debrisoquine was a competitive inhibitor of the 1′-hydroxylation of both isomers and of the racemate of bufuralol.

4. Both isomers and the racemate of bufuralol were competitive inhibitors of debrisoquine 4-hydroxylase activity.

5. The competitive inhibition of debrisoquine and bufuralol of each other's metabolism, together with the similarity in the values for K_m and K_i, support the conclusion that the same form of cytochrome P-450 catalyses these two reactions.

A genetic polymorphism of drug oxidation in man, discovered with debrisoquine [1] and sparteine [2] has been shown to affect a number of other drugs [3]. In addition, an unrelated polymorphism in the oxidation of mephenytoin has been described [4]. It is widely assumed that impaired metabolism of these drugs is due to the absence or alteration of a form(s) of hepatic cytochrome P-450. Evidence in support of this assumption has been presented for the impaired oxidation of debrisoquine [5]. In these studies liver from a poor metabolizer (PM)† phenotype had no measurable debrisoquine 4-hydroxylase activity. Liver samples from the PM phenotype, who comprise less than 10% of the population [6], are difficult to obtain. In addition, such studies with a range of substrates, although demonstrating that the PM liver has impaired oxidation, do not prove that the same form of cytochrome P-450 is involved in the oxidation of all these compounds. Another approach is to study the kinetics of inhibition of compounds believed to be competing substrates for the affected form of cytochrome P-450.

We have shown previously that drugs whose metabolism is impaired in PM subjects are potent competitive inhibitors of debrisoquine 4-hydroxylase activity *in vitro* whereas compounds whose metabolism is not affected in poor metabolizers are either weak, non-competitive inhibitors, or do not inhibit activity at all [7]. Competitive inhibition indicates that a drug is able to bind to the same active site of cytochrome P-450 catalysing the oxidation of debrisoquine. It does not prove that this form of cytochrome P-450 oxidises the drug. A further study of the effects of debrisoquine on the oxidation of the drug is required.

Amongst compounds whose oxidation has recently been reported to be impaired in the poor metabolizer phenotype is the β-adrenoceptor antagonist bufuralol, for which the 1′-hydroxylase activity is affected [8] (Fig. 1). Bufuralol is optically active, and there have been reports, based on *in vivo* studies, that the oxidation of the isomers of another optically active β-adrenoceptor antagonist, metoprolol, is differentially affected in the debrisoquine oxidation polymorphism [9].

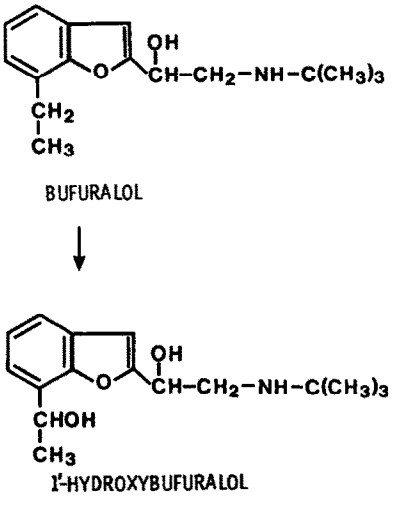

Fig. 1. Structure of bufuralol and its hydroxylated metabolite 1′-hydroxybufuralol.

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† Abbreviations used: HPLC, high pressure liquid chromatography; EM and PM phenotype, extensive and poor metabolizer phenotype respectively as defined in [1].

In the present investigation we have examined the inhibition of debrisoquine 4-hydroxylase by the isomers of bufuralol and the effects of debrisoquine on the 1′-hydroxylation of bufuralol.

MATERIALS AND METHODS

Materials. Debrisoquine hemisulphate, 4-hydroxydebrisoquine hemisulphate, (±)bufuralol hydrochloride, (+)bufuralol hydrochloride, (−)bufuralol hydrochloride and 1′-hydroxybufuralol were all generous gifts of Roche Products plc (Welwyn Garden City, U.K.). [$^2H_9$]4-Hydroxydebrisoquine was prepared biologically as previously described [10]. NADPH (tetrasodium salt, type I) and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Hexafluoroacetylacetone was obtained from Fluorochem Ltd. (Glossop, Derbyshire, U.K.). Methyl-*t*-butyl ether and acetonitrile, both of HPLC grade, were purchased from Rathburn Chemicals (Walkerburn, Scotland). All other reagents were of Analar grade. Toluene was redistilled before use.

Human liver samples. Human liver samples were obtained from renal transplant donors who had met accidental deaths and who were maintained on life support systems until such time as the kidneys were removed. At this time liver samples were also taken. Local Research Ethics Committee permission and coroner's approval were obtained to use such tissue in these studies. The tissue was homogenized and fractionated by ultracentrifugation as previously described [11] and the microsomal suspensions were stored in 0.25 M potassium phosphate buffer, pH 7.25, containing 30% glycerol at −80° until required. In previous studies it had been established that neither storage of the tissue in this way, nor the fact that the liver samples were from renal transplant donors, resulted in any loss of activity of the debrisoquine 4-hydroxylase form of cytochrome P-450 [10].

Bufuralol 1′-hydroxylase activity. The incubation mixture, in a total volume of 0.25 ml, comprised sodium phosphate buffer, pH 7.4, 5 mM magnesium chloride, 2.4 mM NADPH and 0.2 mg microsomal protein. The reaction was started by the addition of bufuralol, dissolved in water at 2.5 times the desired final concentration. Samples were incubated at 37° in a shaking water bath in air for 20 min. The reaction was terminated by the addition of 10 μl of 5 M sodium hydroxide and transferring the samples on to ice. This was followed by the addition of 2 ml of methyl-*t*-butyl ether into which 1′-hydroxybufuralol was extracted by vortex mixing the tubes for 15 sec. The phases were separated by centrifugation at 1000 *g* for 10 min and the organic phase transferred for HPLC analysis.

1′-Hydroxybufuralol was quantitated by HPLC-fluorescence spectrometry, using straight phase chromatography. The stationary phase was Spherisorb 5 μm silica (Shandon Southern Products Ltd, Runcorn, Cheshire, U.K.) packed in a 25 cm × 4 mm stainless steel column. The mobile phase was 80% methyl-*t*-butyl ether, 20% acetonitrile containing 0.04% perchloric acid. This was delivered at a flow rate of 2.5 ml/min by twin Altex model 100A pumps controlled by a model 420 programmer (Anachem Ltd, Luton, Beds, U.K.). The injection volume, via a Rheodyne loop injector (Anachem Ltd, Luton, Beds, U.K.), was 200 μl. Detection of 1′-hydroxybufuralol was by fluorescence spectrometry (Model LS4, Perkin-Elmer Ltd, Beaconsfield, Bucks, U.K.) with excitation wavelength of 244 nm and emission wavelength of 312 nm. Both excitation and emission slits were set to 10 mm. Quantitation was from peak height, recorded on a Tekman dual pen potentiometric recorder (Anachem, Luton, Beds, U.K.) with reference to external standards taken through the normal incubation procedure but to which substrate was not added until after the addition of the alkali and methyl-*t*-butyl ether. The identity of the peak eluting from incubated samples at the position of authentic 1′-hydroxybufuralol was confirmed by gas chromatography–mass spectrometry.

Other assays. Microsomal protein content [11] with bovine serum albumin, fraction V, as standard and debrisoquine 4-hydroxylase activity [7] were determined as previously described.

In all inhibition studies the inhibitor, dissolved in water at twice the final concentration, was added to the complete incubation mixture in half the final incubation volume, prior to the addition of substrate. The samples were then preincubated at 37° for 2 min before starting the reaction by addition of the substrate.

Analysis of the results. The data were analysed using iterative non-linear least squares regression analysis procedures as previously described [7]. Bufuralol 1′-hydroxylase activity was monophasic over the range of substrate concentrations used, and so was analysed assuming a single enzyme system. As previously reported [7], debrisoquine 4-hydroxylase activity of human liver has two components. The data for this enzyme were therefore analysed assuming a 4-parameter, two enzyme system. Inhibitor constants, K_i values, were calculated as previously described [7].

RESULTS

The HPLC-fluorescence spectrometric assay for 1′-hydroxybufuralol is capable of detecting 250 pg (0.83 pmole) of the metabolite on column. The recovery of 1′-hydroxybufuralol through the extraction procedure was 85 ± 3% (mean ± S.D., N = 6). The precision of the assay was 2.5% with an inter-assay variation of 5.5% at a substrate concentration of 25 μM. Enzyme activity was linear with time to at least 40 min and with microsomal protein to at least 2 mg/ml. The conditions of the assay were thus well within the linear range for these two variables. The kinetics of bufuralol 1′-hydroxylase activity were monophasic over the range of substrate concentrations investigated (0–100 μM). Michaelis–Menten parameters for the 1′-hydroxylation of the isomers of bufuralol and for the racemic mixture are shown in Table 1. V_{max} for (+)bufuralol was 153 pmole/mg/min and was three times that for the (−)isomer which was 52 pmole/mg/min. The K_m for the (+)isomer, 18.7 μM, was also three times that for (−)bufuralol, at 6.4 μM. V_{max} and K_m for the

Table 1. Kinetics of the 1′-hydroxylation of (+), (−) and (±)bufuralol by microsomal fractions of human liver

Substrate	V_{max} (pmole/mg/min)	K_m (μM)
(±)Bufuralol	95.5 ± 3.6*	12.8 ± 1.4
(+)Bufuralol	153.0 ± 8.8	18.7 ± 2.8
(−)Bufuralol	52.0 ± 2.1	6.4 ± 1.0

* Values are the mean ± S.E. of the estimates.

racemate were intermediate to the corresponding values for the individual isomers.

Debrisoquine was a competitive inhibitor of the 1′-hydroxylation of both isomers and the racemate of bufuralol (Fig. 2). There was a dose-dependent increase in K_m for the 1′-hydroxylation of bufuralol with increasing dose of debrisoquine. V_{max} remained almost unaltered (Table 2).

The effects of the isomers of bufuralol on debrisoquine 4-hydroxylase activity were investigated. Both isomers of bufuralol and its racemate were potent competitive inhibitors of the 4-hydroxylation of debrisoquine (Fig. 3). There was no change in V_{max} with any of the forms of bufuralol, but K_m increased with increasing concentration of the inhibitor (Table 3). The K_i values calculated with the different concentrations of inhibitor were in good agreement with each other (data not shown). There was excellent agreement between the K_i values for the inhibition of debrisoquine 4-hydroxylase activity and the K_m for the 1′-hydroxylation of the corresponding isomer of bufuralol (Table 4). The (−)isomer was more than three times as potent an inhibitor as the (+)isomer and the racemate was of intermediate potency.

Table 3. Effect of the isomers of bufuralol on the kinetics of debrisoquine 4-hydroxylase activity

Inhibitor	Concentration (μM)	Debrisoquine 4-hydroxylase activity V_{max} (pmole/mg/min)	K_m (μM)
(±)Bufuralol	0	59.1*	164
	10	61.2	260
	25	59.0	453
(+)Bufuralol	0	48.6	111
	5	49.0	130
	25	57.5	232
(−)Bufuralol	0	55.9	132
	5	61.5	230
	25	53.4	605

* Values have been corrected for the contribution of the low affinity component of debrisoquine 4-hydroxylase activity as described under Materials and Methods.

DISCUSSION

An HPLC assay, utilizing fluorescence detection, has been developed to investigate the 1′-hydroxylation of bufuralol by microsomal fractions of human liver. The assay is sensitive, specific and reproducible, with a high recovery of the metabolite. There is good linearity with both the amount of microsomal protein in the incubation and the duration of incubation.

As reported for many optically active substrates for the cytochrome P-450 dependent monooxygenase system, such as warfarin and propranolol, the metabolism of the isomers of bufuralol proceeds at different rates. The V_{max} for the (+)isomer was three times that of the (−)isomer. However, the K_m for the (+) isomer was also three times that of the (−)isomer. As predicted from the Michaelis–Menten parameters for the individual isomers, the V_{max} and K_m for the 1′-hydroxylation of the racemate of bufuralol are intermediate to the corresponding values for the individual isomers (Table 5).

If it is assumed that each isomer acts as a competitive inhibitor of the 1′-hydroxylation of the other isomer of bufuralol, it can be calculated from the Michaelis–Menten parameters for the individual isomers that the (+)isomer of bufuralol will contribute approximately 50% to total 1′-hydroxylase activity at

Table 2. The effects of debrisoquine on the 1′-hydroxylation of the isomers of bufuralol

Substrate	Concentration of debrisoquine (μM)	Bufuralol 1′-hydroxylase activity V_{max} (pmole/mg/min)	K_m (μM)
(±)Bufuralol	0	95.5 ± 3.6*	12.8 ± 1.4
	250	125 ± 19	86.5 ± 22.2
	500	141 ± 20	154 ± 31
(−)Bufuralol	0	52.0 ± 2.1	6.4 ± 1.0
	250	66.7 ± 3.8	49.8 ± 5.4
	500	68.4 ± 4.0	101 ± 10
(+)Bufuralol	0	153 ± 9.0	18.7 ± 2.8
	250	163 ± 7.0	79.7 ± 5.9
	500	168 ± 7.3	136 ± 9.0

* Values are mean ± S.E.M. of estimates.

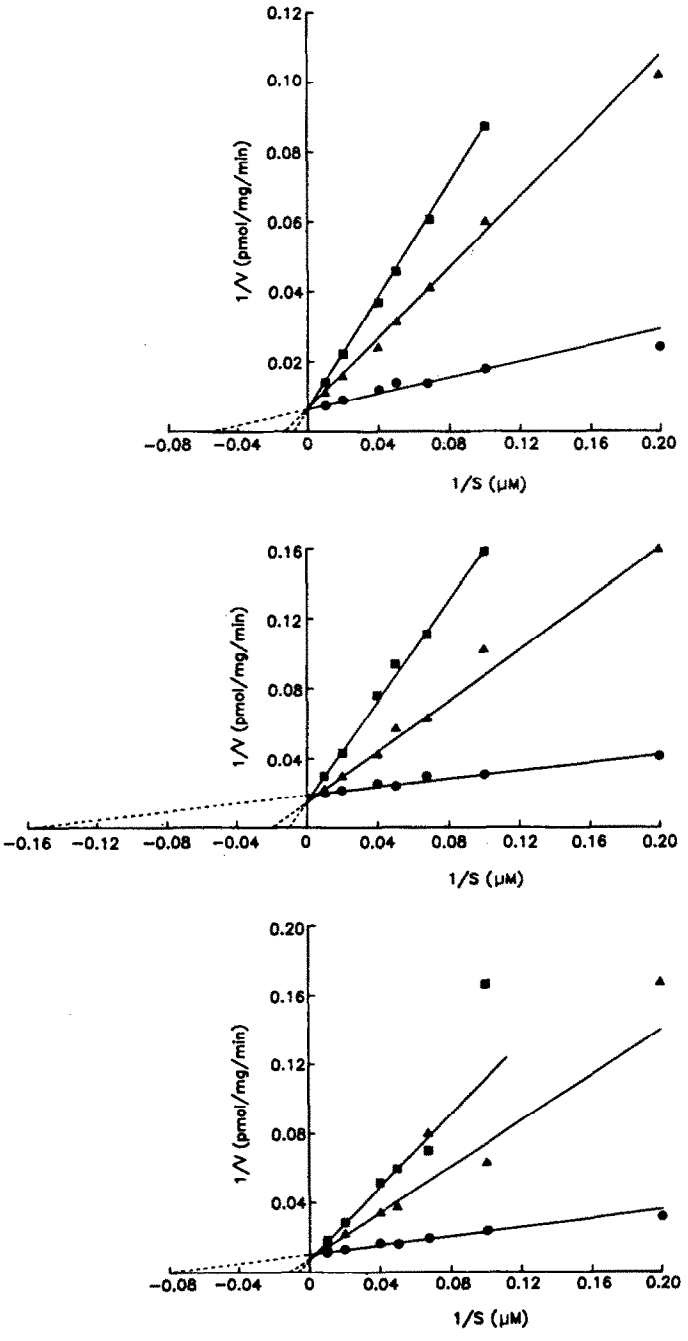

Fig. 2. Effects of debrisoquine on the 1′-hydroxylation of bufuralol by human liver. Activity was determined with (+)bufuralol (top), (−)bufuralol (centre) and (±)bufuralol (bottom) as substrate. Incubations were performed in the absence (●), and in the presence of 0.25 mM (▲) and 0.5 mM (■) debrisoquine. The points show the experimentally derived data and the *solid lines* were fitted by computer as described in Materials and Methods.

all concentrations of the racemic substrate (Table 6).

Bufuralol is a potent competitive inhibitor of debrisoquine 4-hydroxylase activity of human liver. There is excellent agreement between the K_i for inhibition and the K_m for the 1′-hydroxylation of the isomers and the racemate of bufuralol. This suggests that the same form of cytochrome P-450 can catalyse the two reactions, although other forms of cytochrome P-450, with similar K_m values, could be contributing to the 1′-hydroxylation of bufuralol. However, debrisoquine is a potent competitive inhibitor of the 1′-hydroxylation of both isomers and the racemate of bufuralol. This pattern of reciprocal inhibition suggests that there is indeed only one major form of cytochrome P-450 catalysing the two reactions, and that any contribution from other forms of the enzyme must be relatively minor.

The low K_i value for the inhibition of the 1′-

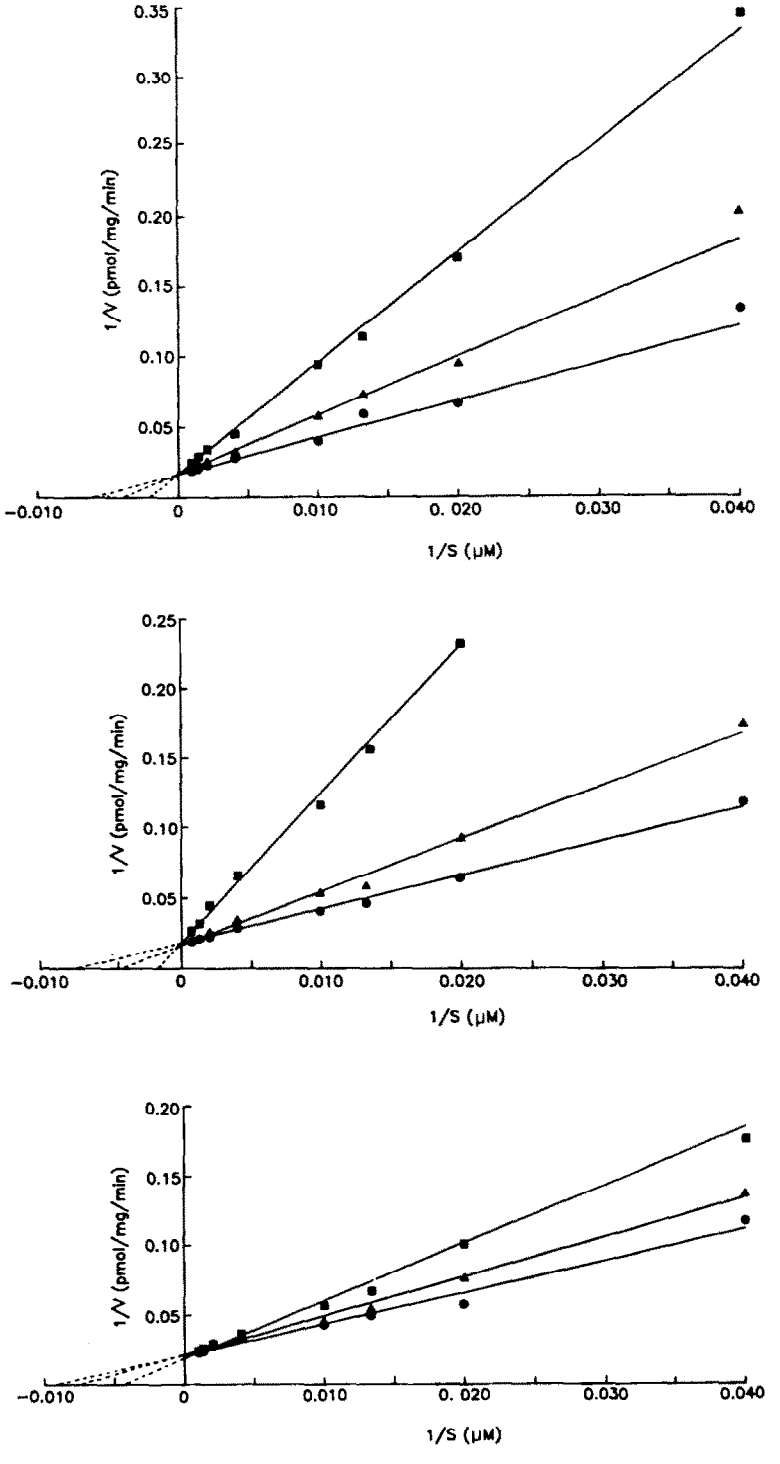

Fig. 3. Effects of the isomers of bufuralol on the 4-hydroxylation of debrisoquine by human liver. Top (±)bufuralol, centre (−)bufuralol and bottom (+)bufuralol were added at concentrations of (●) 0 μM, (▲) 5 μM (10 μM with (±)bufuralol) and (■) 25 μM. The points are the experimentally obtained data corrected for the contribution of the low affinity component as described under Materials and Methods, and the *solid line* is the computer generated best fit, also as described under Materials and Methods.

Table 4. Comparison of K_m and K_i values for debrisoquine and bufuralol

Substrate	K_m (μM)	Inhibitor	K_i (μM)
(±)Bufuralol	12.8	Debrisoquine	44.5
(+)Bufuralol	18.7	Debrisoquine	78.2
(−)Bufuralol	6.4	Debrisoquine	35.4
Debrisoquine	164	(±)Bufuralol	15.6
Debrisoquine	111	(+)Bufuralol	26.0
Debrisoquine	132	(−)Bufuralol	6.9

The values shown under K_m are the Michaelis–Menten constants for the oxidation of the substrate shown in column 1 in the absence of any inhibitor. The values shown under K_i are the inhibitor constants for the inhibitor shown in column 3 on the oxidation of the substrate shown in column 1.

Table 5. Observed and predicted values for the Michaelis–Menten parameters for (±)bufuralol 1′-hydroxylase activity

	Bufuralol 1′-hydroxylase activity	
Activity	V_{max} (pmole/mg/min)	K_m (μM)
Observed	95.5 ± 3.6	12.8 ± 1.4
Predicted	77.8	9.6

Observed values for V_{max} and K_m were calculated from experimental data obtained with (±)bufuralol as substrate. Predicted values were derived from the Michaelis–Menten parameters for the (+) and (−) isomers of bufuralol as described in the text.

hydroxylation of bufuralol by debrisoquine, relative to its K_m, remains unexplained. However, it is perhaps significant that the K_i for inhibition of sparteine oxidation by debrisoquine is also lower than the K_m for the 4-hydroxylation reaction [12], and is similar to the K_i value reported here for inhibition of bufuralol 1′-hydroxylation. The competitive nature of the inhibition of both sparteine and bufuralol metabolism by debrisoquine, together with the similarity in the respective K_i values, is further evidence that the same form of cytochrome P-450 catalyzes the oxidation of sparteine and bufuralol.

It has been reported that the oxidation of the isomers of metoprolol is differentially affected in the debrisoquine oxidation polymorphism [9]. The ratio of oxidation products of the (+)isomer to the (−) isomer is greater than 1 in the EM phenotype and less than 1 in the PM phenotype [9]. One explanation for this observation is stereoselective oxidation by the form of cytochrome P-450 affected in the polymorphism. However, it is also possible that competing pathways of metabolism, unimpaired in the PM phenotype, are stereoselective. With bufuralol as substrate there is no evidence of stereoselectivity in the specificity of the form of cytochrome P-450 catalysing the 4-hydroxylation of debrisoquine. Both isomers of bufuralol inhibit debrisoquine 4-hydroxylase activity with K_i values almost identical to the K_m values for this 1′-hydroxylation. Further, debrisoquine is a competitive inhibitor of the 1′-hydroxylation of both isomers.

It can be predicted from the present data that no difference in the ratio of enantiomers of the metabolites of bufuralol would be observed *in vivo* between the phenotypes. Indeed, recent studies by Dayer *et al.* (personal communication) indicate that the isomeric ratio of the (+)carbinol of bufuralol to the (−)isomer in urine is greater than 1.0 in both phenotypes.

The present study has established that the form of cytochrome P-450 catalysing the 4-hydroxylation of debrisoquine is also the major form catalysing the 1′-hydroxylation of both the (+) and (−) isomers of bufuralol. It seems likely that the previously reported impairment of this form of cytochrome P-450 in poor metabolizers of debrisoquine [5] is responsible for the reduced 1′-hydroxylation of bufuralol in these subjects.

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Table 6. Contribution of the (+) and (−) isomers of bufuralol to the 1′-hydroxylation of a racemic mixture

	Bufuralol 1 -hydroxylase activity (pmole/mg/min)					
Concentration of bufuralol (μM)	Observed velocities (+)Bufuralol	Observed velocities (−)Bufuralol	Predicted contribution in racemate from (+)Bufuralol	Predicted contribution in racemate from (−)Bufuralol	Predicted velocity (±)Bufuralol	Observed velocity (±)Bufuralol
2.5	18.0	14.6	8.1	8.0	16.1	15.6
5	32.3	22.8	13.4	13.3	26.7	26.8
10	52.3	31.7	20.0	19.8	39.8	41.9
20	99.1	29.4	26.4	26.2	52.6	58.2
50	111.4	46.1	32.8	32.5	65.3	76.0
100	128.9	48.9	35.6	35.4	71.0	84.7
200	139.9	50.4	37.2	37.0	74.2	89.8

Predicted velocities were calculated assuming that the racemic mixture comprised equal concentrations of the two isomers and that each isomer acted as a competitive inhibitor of the 1′-hydroxylation of the other. Predicted velocity for (±)bufuralol was calculated from the sum of the predicted contribution from the (+) and (−) isomer at each concentration.

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