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Bufuralol Hydroxylation by Cytochrome P450 2D6 and 1A2 Enzymes in Human Liver Microsomes

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SUMMARY

Bufuralol 1′-hydroxylation is a prototypical reaction catalyzed by cytochrome P450 (P450) 2D6, an enzyme known to show debrisoquine/sparteine-type genetic polymorphism in humans. In the present study we further examined the roles of several human P450 enzymes, as well as P450 2D6, in the hydroxylation of (±)-bufuralol, using liver microsomes from several human samples and human P450 enzymes expressed in human lymphoblastoid cell lines or *Escherichia coli*. Kinetic analysis of bufuralol 1′-hydroxylation by liver microsomes showed that there were different K_m and V_{max} values in seven human samples examined; low K_m values (~0.05 mM) were observed in four samples (including sample HL-18), high K_m values (~0.25 mM) in two samples (including sample HL-67), and an intermediate K_m value (~0.1 mM) in one sample. Quinidine and anti-rat P450 2D1 antibody almost completely inhibited bufuralol 1′-hydroxylation in human sample HL-18 at a substrate concentration of 0.4 mM, whereas these effects were not so drastic when liver microsomes from human sample HL-67 were used. In contrast, a very low concentration (<10 μM) of α-naphthoflavone or anti-human P450 1A2 antibody significantly inhibited bufuralol 1′-hydroxylation catalyzed by human sample HL-67, but not HL-18, with 0.4 mM bufuralol. When the relative contents of P450 2D6 and P450 1A2 in 20 human samples were determined, bufuralol 1′-hydroxylation in samples containing large amounts of P450 2D6 tended to be more sensitive to quinidine, whereas the P450 1A2-rich samples were highly susceptible to α-naphthoflavone. However, at low substrate concentrations bufuralol 1′-hydroxylation was shown to be catalyzed principally by P450 2D6, based on the inhibitory effects of anti-rat P450 2D1 antibody and quinidine, in both human samples HL-18 and HL-67. At least five other, minor, bufuralol products were formed by human liver microsomes, in addition to 1′-hydroxybufuralol. Two of them were identified as 4- and 6-hydroxybufuralol by ^{1}H NMR spectroscopy and mass spectrometry. The formation of the 4- and 6-hydroxylated products was suggested to be catalyzed by P450 1A2, based on the results of correlation with P450 1A2 contents in 60 human samples and inhibition by anti-P450 1A2 and α-naphthoflavone. Purified recombinant P450 1A2 (expressed in *E. coli*) produced 1′-, 4-, and 6-hydroxybufuralol in a reconstituted system, although P450 2D6 (expressed in human lymphoblast cell lines) was found to catalyze only bufuralol 1′-hydroxylation. The K_m value for bufuralol 1′-hydroxylation by recombinant P450 2D6 was estimated to be 0.018 mM; this value was very low, compared with the K_m value of 0.21 mM for recombinant P450 1A2. These results suggest that P450 2D6 is the major catalyst of bufuralol 1′-hydroxylation, although P450 1A2 is also active in the hydroxylation reactions in humans and contributes considerably to 4- and 6-hydroxybufuralol formation. These phenolic products are known to be present in human urine and may find use in noninvasive measurements.

Genetic polymorphism in the oxidative metabolism of clinically used drugs is one of the critical factors for understanding the basis of different susceptibilities of individuals to pharmacological and toxicological actions of these drugs (1, 2). Debrisoquine/sparteine-type genetic polymorphism is one of the best studied P450-dependent polymorphisms in humans and has been identified to be due to genetic defects in the *CYP2D6* gene in the poor-metabolizer phenotype (3, 4). About 7% of the Caucasian population has been identified to be of the poor-metabolizer phenotype, whereas a very low incidence of genetic defects has been observed in Japanese and Chinese populations (5). A number of variant alleles associated with the poor-metabolizer phenotype have been described in Caucasian populations, and several mechanisms have been proposed to account for the polymorphism (4, 6).

Bufuralol (Fig. 1), a β-adrenergic receptor antagonist, is reported to be metabolized to 1′-hydroxybufuralol (M-1) by P450 2D6 and therefore has been used widely *in vitro* for the study of debrisoquine/sparteine-type genetic polymorphism in humans (7, 8). In 1986 Gut *et al.* (7) reported that there are two different forms of human P450 2D-related proteins, "buf I" and "buf II," involved in bufuralol 1′-hydroxylation in human liver microsomes. The former enzyme is now called P450 2D6, whereas the nature of the second protein, buf II, has not been

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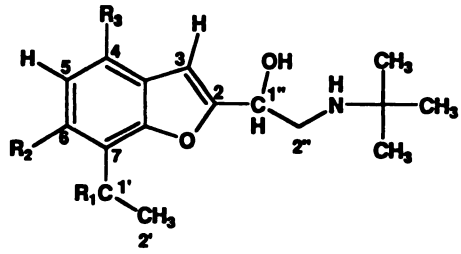
ABBREVIATIONS: P450, cytochrome P450; ANF, α-naphthoflavone; HPLC, high performance liquid chromatography.



		R_1	R_2	R_3
	Bufuralol	H,H	H	H
M-1	1'-Hydroxybufuralol	H,OH	H	H
M-2a	6-Hydroxybufuralol	H,H	OH	H
M-2b	1'-Oxobufuralol	O	H	H
M-3	4-Hydroxybufuralol	H,H	H	OH

Fig. 1. Structures of bufuralol and its oxidation products.

characterized. In the course of our studies on the characterization of interindividual variations in various forms of P450, however, we found that in some of the human samples the bufuralol 1'-hydroxylation activities of liver microsomes could not be inhibited substantially by anti-rat P450 2D1 or the specific P450 2D6 inhibitor quinidine, whereas in other cases the activity was inhibited very strongly. These results suggest that other P450 enzymes, as well as P450 2D6, may catalyze bufuralol 1'-hydroxylation in liver microsomes of some of the human samples. Recently we have obtained evidence in rat liver microsomes suggesting that P450 1A1, 1A2, and 2C11 can catalyze bufuralol 1'-hydroxylation, although P450 2D1 has a dominant role because of its high affinity for the substrate in rat liver microsomes (9).

In this study, therefore, we further examined the roles of different human P450 enzymes in the hydroxylation of (±)-bufuralol by utilizing liver microsomes from different human samples and also human P450 enzymes expressed in human lymphoblastoid cell lines or *Escherichia coli*. HPLC analysis showed that several oxidation products of bufuralol were formed with liver microsomes from different human samples; the formation of M-2 as well as 1'-hydroxybufuralol by rat liver microsomes has also been reported in our laboratories recently (9). We report here the catalytic roles of several human P450 enzymes in the formation of these bufuralol metabolites in different human samples. The results obtained suggest that the relative contents of individual forms of P450 in different human samples and the kinetics of P450 enzymes for the bufuralol hydroxylation activities may be critical factors for understanding the basis for interindividual variations in P450-catalyzed drug hydroxylation reactions in humans.

Experimental Procedures

Chemicals. (±)-Bufuralol and 1'-hydroxybufuralol were gifts from Hoffmann-LaRoche Co. (Nutley, NJ). Other chemicals used were from the same sources described previously (10, 11).

Enzyme preparations and antibodies. Human liver samples were obtained from 30 patients (designated HL-1 to HL-10 and HL-23 to HL-48) undergoing liver resection and from 30 organ donors (designated HL-11 to HL-18 and HL-49 to HL-72), through Tennessee Donor Services (Nashville, TN) (12). Liver microsomes were prepared as described and suspended in 10 mM Tris·HCl buffer, pH 7.4, containing 0.10 mM EDTA and 20% (v/v) glycerol (13). Microsomes of human lymphoblastoid cells expressing human P450 enzymes were prepared as described elsewhere (14, 15).

Human P450 1A2, 2A6, $2C_{MP}$, 2E1, and 3A4 were purified to electrophoretic homogeneity and rabbit anti-P450 antibodies were prepared (11, 16–19). Recombinant human P450 1A2, 2C10, 2E1, and 3A4 proteins were purified from membranes of *E. coli* in which modified P450 genes had been introduced as described (20–23). Rabbit liver NADPH-P450 reductase and cytochrome b_5 were purified by the method of Yasukochi and Masters (24), as modified by Taniguchi *et al.* (25).

Assay methods. Bufuralol 1'-hydroxylation activities of liver microsomal P450 enzymes were determined according to the general method of Kronbach *et al.* (26, 27), with modifications. Incubation mixtures consisted of human liver microsomes (1.0 mg of protein/ml) or microsomes (1.6 mg of protein/ml) from human lymphoblast cell lines and bufuralol (0.40 mM) in a final volume of 0.10 ml of 100 mM potassium phosphate buffer, pH 7.4, containing an NADPH-generating system, as described previously (11). Reconstituted P450 systems were composed of 20–50 nM purified human P450, 40–100 nM cytochrome b_5, 100 nM NADPH-P450 reductase, 10 μg/ml levels of a phospholipid mixture consisting of L-α-dilauroyl-*sn*-glycero-3-phosphocholine, dioleoyl-*sn*-glycero-3-phosphocholine, and L-α-phosphatidyl-L-serine (1:1:1, w/w/w), and 0.25 mM sodium cholate (10). Incubations were carried out at 37° for 30 min and were terminated by addition of 10 μl of 60% $HClO_4$ (w/v). The mixtures were centrifuged at 3000 × *g* for 5 min and aliquots of the supernatant were used for the determination of hydroxylated metabolites by HPLC (model CCPD; Tosoh Co., Tokyo, Japan). The separation was carried out with a 4.6- × 250-mm octadecylsilane reverse phase column (Chromato Tech Co., Tokyo, Japan) eluted with 45% (v/v) CH_3CN/55% 20 mM $NaClO_4$, pH 2.5. The hydroxylated metabolites of bufuralol were detected with a fluorescence detector (model RF-530; Shimadzu, Kyoto, Japan) (excitation, 252 nm; emmission, 302 nm) and UV detector (A_{254}) used in tandem.

Preparation of bufuralol oxidation products for identification. Two approaches were used to prepare large amounts for analysis. Products M-2 and M-3 were isolated from a 36-ml incubation containing 1.0 μM recombinant P450 1A2, 1.0 μM rabbit NADPH-P450 reductase, 30 μM L-α-dilauroyl-*sn*-glycero-3-phosphocholine, 0.10 M potassium phosphate, pH 7.7, 0.20 mM (±)-bufuralol, and 0.2 nM bovine erythrocyte catalase (to prevent destruction by generated H_2O_2) (28). The incubation proceeded for 30 min at 37°, after which it was stopped by the addition of 3.6 ml of 68% (w/v) $HClO_4$. Salt was removed from the chilled (0°) solution by centrifugation (30,000 × *g* for 10 min), and the supernatant was injected (in 5-ml portions) onto a 10- × 250-mm Beckman Ultrasphere octadecylsilane HPLC column (5 μm; Beckman, San Ramon, CA), using the same solvent system employed in the analytical mode. The individual peaks were collected and the volume was reduced *in vacuo*; the pH was raised to >10 with NaOH and each fraction was extracted three times with CH_2Cl_2. The combined CH_2Cl_2 layers in each case were dried with Na_2SO_4, filtered through paper, and concentrated to dryness *in vacuo*.

Spectroscopy. UV spectra were recorded on a Cary 14/OLIS instrument (On-Line Instrument Systems, Bogart, GA), and λ_{max} values were determined using either the peak-finder program or second-derivative analysis. ^1H NMR spectra were obtained with a Bruker AM-400 instrument (Bruker, Billerica, MA), in ^2H_2O or C^2HCl_3; 4,4-dimethyl-4-silapentane-1-sulfonic acid and $(CH_3)_4Si$ were used as standards for aqueous and nonaqueous samples, respectively.

Other assays. P450 was estimated spectrally by the method of Omura and Sato (29). The contents of individual human P450 proteins in human liver microsomes were estimated by coupled sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunochemical development (Western blotting) (17, 30). Protein concentrations were estimated by the method of Lowry *et al.* (31).

Analysis of data and statistical methods. Kinetic parameters for the hydroxylation of bufuralol by human liver microsomal P450 enzymes were estimated using a nonlinear regression analysis program (K·cat; BioMetallics, Princeton, NJ). The correlations between activities of bufuralol hydroxylation and contents of individual forms of P450 in different human liver microsomal preparations were analyzed

using a linear regression analysis program (InStat; GraphPad Software, San Diego, CA). Statistical analysis was performed with Student's t test.

Results

Bufuralol hydroxylation in human liver microsomes. In liver microsomes prepared from human sample HL-18 only 1′-hydroxybufuralol (M-1) was detected (Fig. 2A), whereas two major fluorescent metabolites (1′-hydroxybufuralol as the major metabolite and M-2 as a minor metabolite) were found in human sample HL-67 (Fig. 2B). In another sample known to be relatively low in P450 2D6 and high in P450 1A2 (HL-18), the M-2 and M-3 products were produced in excess of M-1 (substrate concentration, 0.20 mM). P450 1A2 expressed in *E. coli* was also found to catalyze both 1′-hydroxybufuralol and M-2 formation (Fig. 2C) (see below).

Identification of bufuralol oxidation products. A preparative-scale incubation for oxidation of bufuralol was done with purified recombinant P450 1A2, using a UV detector for the HPLC system (Fig. 2D). Several products were obtained, including the known product 1′-hydroxybufuralol (M-1), identified by its retention time and fluorescence. A closely following peak was seen (M-1a), which was also fluorescent but was not identified. Because this peak was not seen in short incubations, it would appear to be a product of secondary oxidation.

Peaks M-2, M-4, and M-5 all showed the characteristic bufuralol fluorescence ($F_{250/300}$) but peak M-3 had little fluorescence under these conditions. The peaks were collected from the column and recovered after extraction into CH_2Cl_2 at basic pH. Peaks M-2 and M-3 showed a broad UV band near 290 nm (as well as stronger bands near 210 and 250 nm), which is probably due to their phenolic character (see below) and clearly differs from 1′-hydroxybufuralol. It should be noted that 1′-oxobufuralol has been reported not to be fluorescent (32).

The ^{1}H NMR spectra of the oxidation products are reported in Fig. 3 and Table 1 (with those of bufuralol and 1′-hydroxybufuralol). Peak M-2 appeared to contain a mixture of 6-hydroxybufuralol (major) and 1′-oxobufuralol (minor). These assignments are based upon decoupling studies, with reference to a previous assignment of 6-hydroxybufuralol (33). Critical to the assignment is the appearance of two coupled (J = 8.3 Hz) doublets (δ 6.74 and 7.20) in the aromatic region, indicative of the change from three coupled vicinal protons to two (Fig. 1). The presence of 1′-oxobufuralol (M-2b) was noted by the apparent loss of the 1′ protons and the appearance of a new singlet at δ 2.10. The mass spectrum of peak M-2 showed a mixture of the parent ions $(M+H)^+$ at m/z 278 (6-hydroxybufuralol; relative abundance, 48) and m/z 276 (1′-oxobufuralol; relative abundance, 18).

The M-3 peak appeared to be chemically pure, as judged by the ^{1}H NMR spectrum (Fig. 3). The mass spectrum showed $(M+H)^+$ at m/z 278 (relative abundance, 30). The ^{1}H NMR spectrum, like that of the main component of product M-2, showed the characteristic pair of coupled vicinal doublets in the aromatic region (Table 1). This information and the precedent of Francis *et al.* (33) lead to the assignment as 4-hydroxybufuralol.

The UV spectra obtained with the material in peaks M-4 and M-5 were rather nondescript and devoid of the 290-nm band; the ^{1}H NMR spectra were weak due to the limited amount of material available. The spectra obtained for peak M-4 were very complex and suggested a mixture; the mass spectra also suggested a mixture, although there was a strong peak at m/z 278. With the material from peak M-5, the mass spectrum showed apparent $(M+H)^+$ peaks at m/z 260 (relative abundance, 44), 276 (relative abundance, 7), and 294 (relative abundance, 29). The ^{1}H NMR spectrum could be deconvoluted with decoupling to show an apparent mixture of two components, which could be partially characterized (Table 1). It appears that the H-4/5/6 aromatic pattern of both is intact. The exact identities of the components are not clear; the mass spectra suggest doubly oxidized products.

Kinetic analysis of bufuralol 1′-hydroxylation by human liver microsomes. Seven human liver microsomal sam-

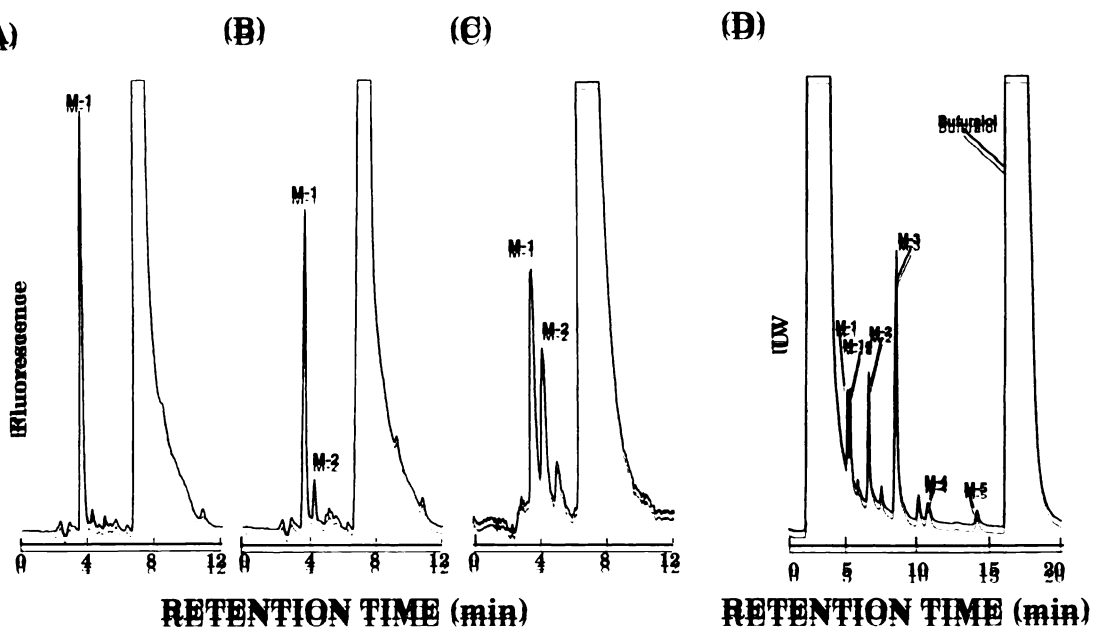


Fig. 2. HPLC analysis (with fluorescence detection) of bufuralol hydroxylation catalyzed by liver microsomes prepared from human samples HL-18 (A) and HL-67 (B) and by a reconstituted monooxygenase system containing purified P450 1A2 expressed in *E. coli* (C) and the HPLC profile (with UV detection, A_{254}) of bufuralol oxidation products formed by recombinant human P450 1A2 (D).

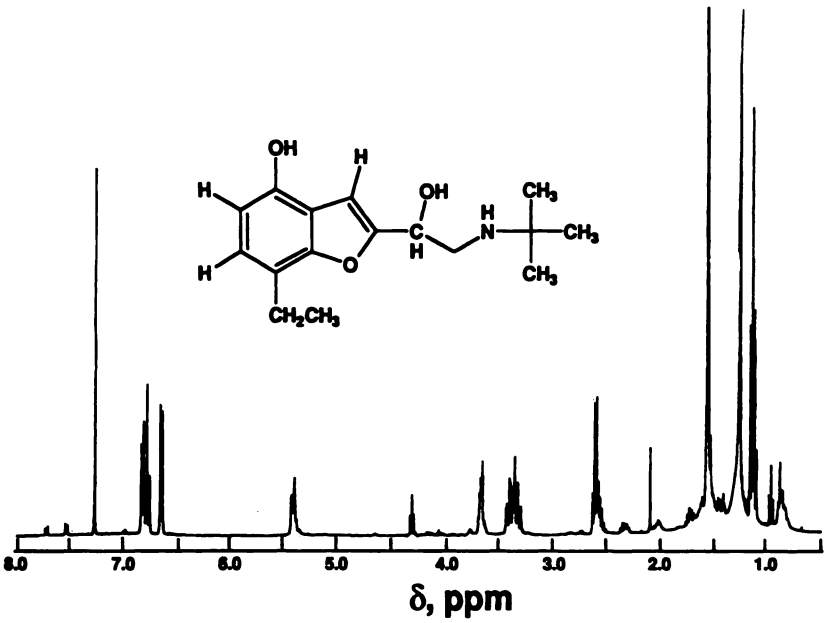


Fig. 3. ^1H NMR spectrum of bufuralol product M-3 (in C^2HCl_3). For assignments and coupling constants, see Table 1.

ples were used to determine the kinetics of bufuralol hydroxylation activities according to the procedures described in Experimental Procedures (Fig. 4). We twice examined the kinetics of bufuralol hydroxylation activities of these human liver microsomes, and we found that the results obtained were basically similar in the two experiments. The K_m and V_{max} values for bufuralol 1′-hydroxylation activities differed among the samples used. The lower K_m values (~0.05 mM) were obtained in human samples HL-13, HL-18, HL-51, and HL-70 and the higher K_m values (~0.25 mM) were determined in two human samples, HL-50 and HL-67. One human sample (HL-53) showed an intermediate apparent K_m value of 0.11 mM. The statistical analysis showed that the K_m values obtained in HL-50 and HL-67 were significantly different ($p < 0.05$) from those for HL-13, HL-18, HL-51, and HL-70. The K_m value obtained in HL-53 was also found to be significantly different ($p < 0.05$) from those for HL-13, HL-18, HL-51, and HL-70. In addition, a low K_m value (for bufuralol 1′-hydroxylation) seemed to be linked to a higher V_{max} value for bufuralol 1′-hydroxylation activity in liver microsomes from different human samples.

Effects of anti-P450 antibodies on bufuralol 1′-hydroxylation catalyzed by human liver microsomes. The results described above suggested that there are different forms of P450 involved in bufuralol hydroxylation by human liver microsomes. To determine the possible roles of individual human P450 enzymes in the reaction, the effects of anti-P450 antibodies were examined in liver microsomes from human samples HL-18 and HL-67, which showed low and high K_m values, respectively, for bufuralol hydroxylation (Fig. 5). At a 0.40 mM substrate concentration, only anti-rat P450 2D1 antibody markedly inhibited bufuralol 1′-hydroxylation with human sample HL-18. With human sample HL-67, however, the effect of anti-P450 2D1 was not so drastic and anti-P450 1A2 was found to inhibit the bufuralol 1′-hydroxylation activity by ~50%. Antibodies raised against P450 2A6, P450 $2C_{MP}$, and P450 2E1 did not inhibit bufuralol 1′-hydroxylation in liver microsomes of human samples HL-18 and HL-67 (data not shown).

The possibility exists that the effects of anti-P450 antibodies might be variable depending on the substrate concentrations used, because different K_m values were observed in several human samples. Bufuralol 1′-hydroxylation in sample HL-18 was inhibited very significantly by anti-P450 2D1 at all of the substrate concentrations used (Fig. 6). In contrast, bufuralol 1′-hydroxylation activity catalyzed by liver microsomes from human sample HL-67 was inhibited very significantly by anti-P450 2D1 at lower substrate concentrations, whereas the effects of anti-P450 2D1 were decreased at the higher substrate concentrations examined.

Effects of quinidine and ANF on bufuralol hydroxylation by human liver microsomes. These results suggested that P450 2D6 and P450 1A2 can both have roles in bufuralol hydroxylation in human liver microsomes. Two P450 inhibitors, ANF for P450 1A enzymes (34, 35) and quinidine for P450 2D6 (36, 37), were used to inhibit bufuralol hydroxylation activities catalyzed by human samples HL-18 and HL-67 (Fig. 7). Quinidine very strongly inhibited bufuralol 1′-hydroxylation in human sample HL-18, whereas it poorly inhibited the activity of human sample HL-67. In contrast, ANF inhibited bufuralol 1′-hydroxylation activity in human sample HL-67 at a very low concentration (IC_{50} of <10 μM), whereas in human sample HL-18 the IC_{50} was >100 μM.

The inhibition constants for inhibition by ANF and quinidine of bufuralol 1′-hydroxylation were determined in liver microsomes from human samples HL-18 and HL-67 (Fig. 8). Quinidine competitively inhibited bufuralol 1′-hydroxylation in liver microsomes from both samples; the K_i values were determined to be 0.78 μM and 8.7 μM for human samples HL-18 and HL-67, respectively. ANF was found to inhibit bufuralol hydroxylation and the K_i value was determined to be ~11 μM in liver microsomes from both human samples.

To determine whether ANF inhibits P450 2D-dependent drug hydroxylation activities as well as P450 1A2-catalyzed reactions, we examined the effects of ANF on bufuralol hydroxylation by microsomes of human lymphoblast cell lines expressing human P450 2D6, by a reconstituted monooxygenase system containing rat P450 2D1, and by liver microsomes from untreated male rats (Fig. 9). ANF (50 μM) inhibited P450 2D6-supported bufuralol 1′-hydroxylation activities by ~50%, whereas the rat P450 2D1-catalyzed reaction was not affected

TABLE 1
NMR chemical shifts, coupling constants, and assignments for bufuralol and P450 1A2 oxidation products

	Proton assignment										
	3	4	5	6	1′	2′	1″	2a″	2b″	tert-Butyl
	δ									
Bufuralol	6.70 (s)	7.36 (dd; J = 0.94, 7.5)[a]	7.15 (t; J = 7.5)	7.10 (d; J = 6.9)	2.92 (q; J = 7.6)	1.33 (t; J = 7.6)	5.06 (dd; J = 3.9, 7.9)	3.22 (dd; J = 3.9, 12.1)	3.15 (dd; J = 7.9, 12)	1.28 (s)
M-1	6.73 (s)	7.30 (d; J = 7.3)	7.21 (t; J = 7.5)	7.43 (d; J = 7.6)	5.35 (m, b)	1.63 (d; J = 5.3)	5.55 (m, b)	3.47 (m, b)	3.33 (m, b)	1.52 (s)
M-2a	6.77 (s)	6.74 (d; J = 8.3)	7.20 (d; J = 8.3)		2.88 (q; J = 7.8)	1.45 (t; J = 7.3)		3.68	3.68	1.53 (s)
M-2b	6.70 (s)	7.88 (dd; J = 1.2, 7.1)	7.31 (t; J = 7.7)	7.66 (dd; J = 1.1, 7.8)		2.10 (s)	5.44 (m; J = 8.8)	3.47 (m)	3.38 (m)	1.53 (s)
M-3	6.78 (s)		6.64 (d; J = 8.0)	6.82 (d; J = 8.0)	2.59 (q; J = 7.5)	1.14 (t; J = 7.6)	5.41 (dd; J = 2.4, 11)	3.40 (dd; J = 2.6, 12)	3.34 (dd; J = 7.5, 12)	1.26 (s)
M-5a	6.83 (s)	7.46 (dd; J = 0.95, 7.7)	7.22 (t; J = 7.6)	7.34 (d; J = 7.0)			5.53 (d; J ~ 8)	4.29 (dd; J = 4.3, 12.0)	4.14 (dd; J = 5.9, 11.8)	
M-5b		5.50 (d; J = 11.3)	6.14 (d; J = 17.8)	6.98 (dd; J = 17.8)						

[a] J values are in Hz.

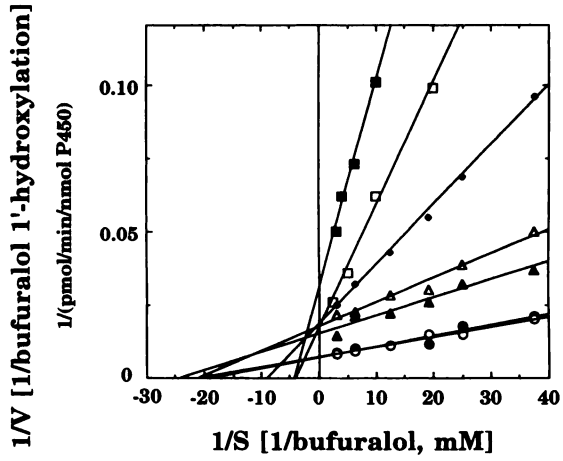


Fig. 4. Kinetic analysis of bufuralol 1′-hydroxylation by liver microsomes from seven human samples. Human liver microsomes (0.10 mg of protein) were incubated with varying concentrations of bufuralol and the products formed were analyzed by HPLC (all V_{max} values are expressed as pmol of product formed/min/nmol of P450). △, HL-13, V_{max} = 54 ± 2 pmol/min/nmol, K_m = 41 ± 2 μM; ●, HL-18, V_{max} = 134 ± 10 pmol/min/nmol, K_m = 44 ± 10 μM; □, HL-50, V_{max} = 69 ± 4 pmol/min/nmol, K_m = 309 ± 35 μM; ▲, HL-51, V_{max} = 77 ± 6 pmol/min/nmol, K_m = 56 ± 11 μM; ◇, HL-53, V_{max} = 53 ± 1 pmol/min/nmol, K_m = 104 ± 5 μM; ■, HL-67, V_{max} = 36 ± 4 pmol/min/nmol, K_m = 268 ± 63 μM; ○, HL-70, V_{max} = 144 ± 4 pmol/min/nmol, K_m = 50 ± 4 μM.

by ANF. As reported previously (9, 36, 37), quinidine was a selective inhibitor of P450 2D6 in humans, whereas quinine was very inhibitory for rat P450 2D1-catalyzed bufuralol 1′-hydroxylation in liver microsomes prepared from untreated male rats.

To determine whether P450 1A2/P450 2D6 levels in liver microsomes are critical factors for explaining the roles of the two P450 enzymes in bufuralol hydroxylation, we compared the P450 levels and effects of 50 μM quinidine and 13 μM ANF in 20 human samples (Fig. 10). ANF was found to be more inhibitory with the human samples high in P450 1A2, and the reverse was the case for the effects of quinidine (i.e., it inhibited more strongly when the P450 1A2/P450 2D6 ratio was decreased).

Bufuralol hydroxylation by liver microsomes from 60 human samples. Sixty human samples were used to determine the activities of bufuralol hydroxylation and to compare these activities with the contents of individual P450 forms and the activities of known P450-catalyzed reactions in these microsomal preparations (Fig. 11). There was considerable variation in bufuralol 1′-hydroxylation activities in the human liver microsomes; the highest activity obtained in these samples was ~0.04 nmol/min/mg of protein (in sample HL-18). Of 60 human samples examined, 46 samples showed detectable activities for M-2 formation, although the activities were always lower than the bufuralol 1′-hydroxylation activities in these samples, except in one case (HL-18). We also determined rates of formation of M-3 in 37 human samples using a UV detector and compared these with rates of phenacetin *O*-deethylation and M-2 formation. Rates of formation of M-2 in these liver microsomes correlated with the activities of phenacetin *O*-deethylation (r = 0.72, $p < 0.001$) and M-3 formation (r = 0.69, $p < 0.001$). On the other hand, bufuralol 1′-hydroxylation activity showed good correlation with P450 2D6 content (r = 0.80, $p < 0.001$), as has been observed previously, but did not show good correlation with the contents of other P450 enzymes.

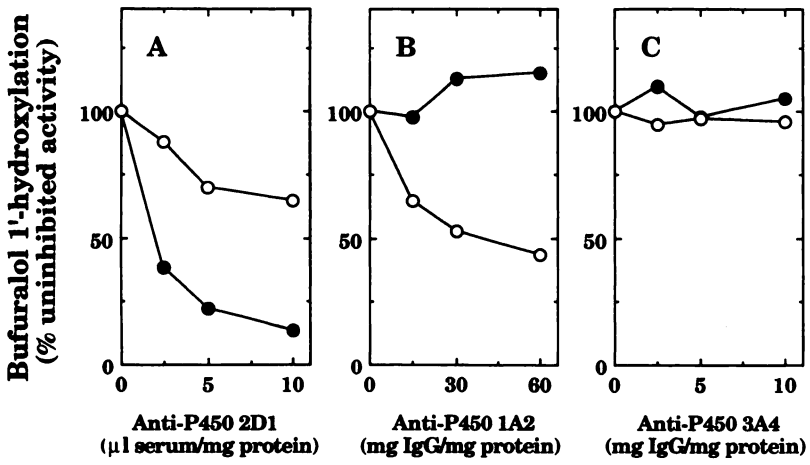


Fig. 5. Effects of anti-rat P450 2D1 serum (A), anti-human P450 1A2 IgG (B), and anti-P450 3A4 IgG (C) on bufuralol hydroxylation catalyzed by liver microsomes prepared from samples HL-18 (●) and HL-67 (○). Preimmune IgG did not affect the hydroxylation activities in either case, and control sera did not cause inhibition when added at the same levels as anti-P450 2D1. Control activities in the absence of antibodies were 170 and 53 pmol/min/nmol of P450 for samples HL-18 and HL-67, respectively.

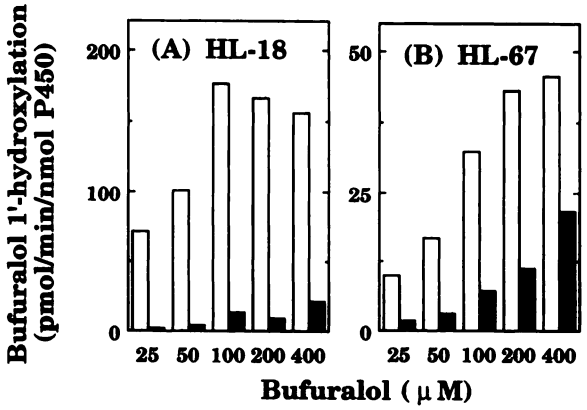


Fig. 6. Effects of substrate concentrations on the inhibition of bufuralol 1'-hydroxylation by anti-rat P450 2D1 in liver microsomes prepared from samples HL-18 (A) and HL-67 (B). Antisera were added to the incubation mixture at 10 μl/mg of microsomal protein. □, No antibodies; ■, with anti-P450 2D1 (preimmune antisera had no effect when added at the same ratio).

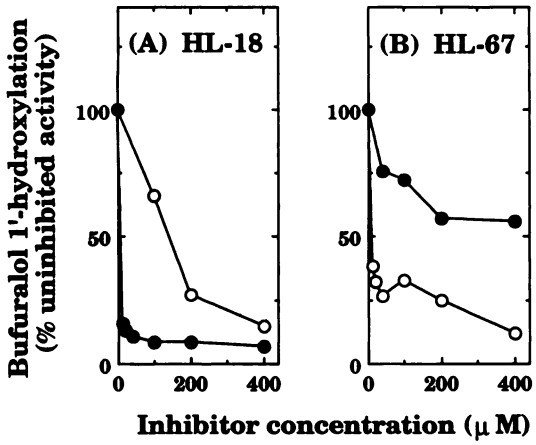


Fig. 7. Effects of ANF (○) and quinidine (●) on bufuralol 1'-hydroxylation catalyzed by liver microsomes prepared from samples HL-18 (A) and HL-67 (B). Control activities in the absence of ANF and quinidine were 156 and 38 pmol/min/nmol of P450 for samples HL-18 and HL-67, respectively.

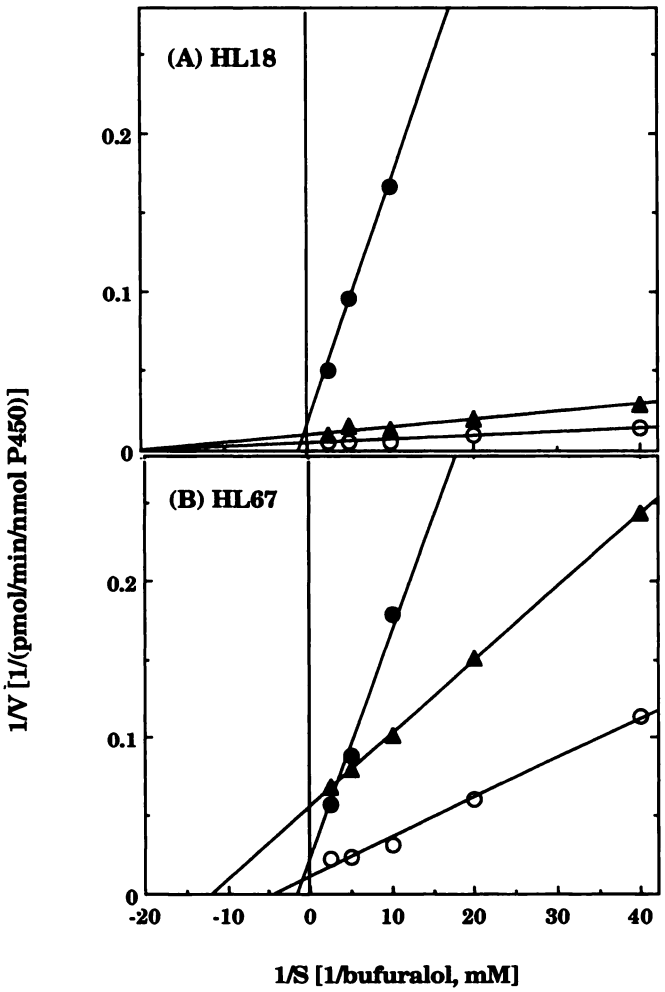


Fig. 8. Inhibition of bufuralol 1'-hydroxylation by ANF and quinidine in human liver microsomes prepared from samples HL-18 (A) and HL-67 (B). ○, Without inhibitor; ▲, with 13 μM ANF; ●, with 50 μM quinidine.

Effects of anti-P450 1A2 on the formation of M-2 and M-3 by human liver microsomes. Because these results suggested that formation of the bufuralol metabolites M-2 and M-3 was catalyzed by P450 1A2 in human liver microsomes, the effects of anti-P450 1A2 on the reactions were examined (Fig. 12). Anti-P450 1A2 strongly inhibited the formation of M-2 and M-3 in liver microsomes prepared from sample HL-67. The formation of M-2 and M-3 by liver microsomes was also found to be blocked by <3 μM ANF (data not shown).

Bufuralol hydroxylation activities of purified and recombinant human P450 enzymes. Purified P450 enzymes isolated from membranes of *E. coli* expressing four human P450 cDNAs and microsomes from human lymphoblast cell lines expressing six human P450 enzymes were used to

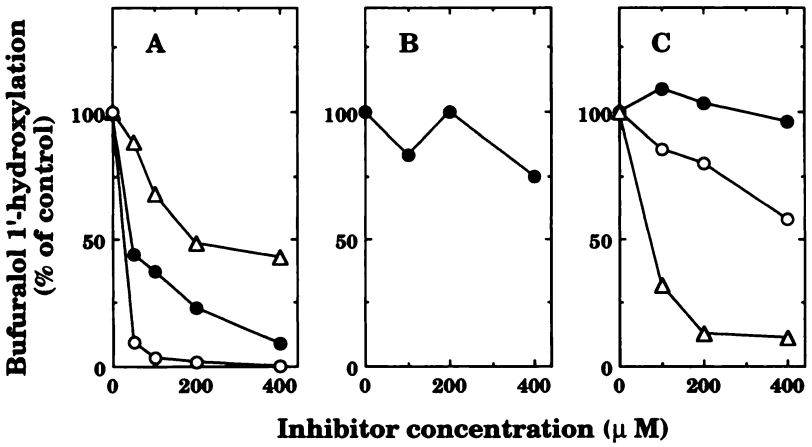


Fig. 9. Effects of ANF (●), quinidine (○), and quinine (△) on bufuralol 1′-hydroxylation catalyzed by P450 2D6 (A), P450 2D1 (B), and liver microsomes prepared from untreated male rats (C). Control activities without chemical inhibitors were 16 pmol/min/mg of protein for P450 2D6, 1050 pmol/min/nmol of P450 for P450 2D1, and 845 pmol/min/nmol of P450 for untreated male rats.

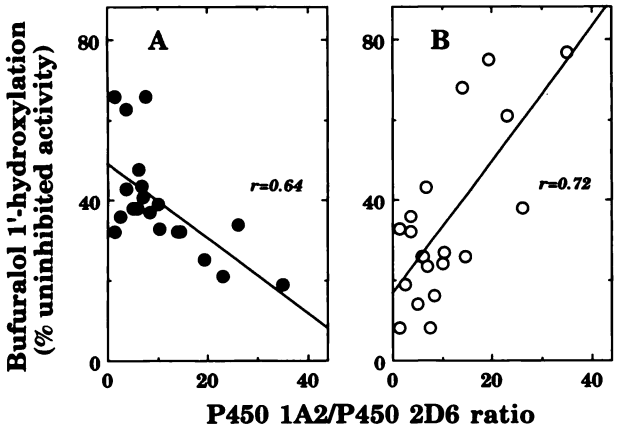


Fig. 10. Effects of 13 μM ANF (A) and 50 μM quinidine (B) on bufuralol 1′-hydroxylation in 20 human liver microsomal samples. *Abscissa*, relative contents of P450 1A2 and P450 2D6 in liver microsomes. Statistically significant; $p < 0.005$ in A and $p < 0.001$ in B.

examine the roles of individual P450 proteins in the bufuralol hydroxylation activities (Fig. 13). In reconstituted monooxygenase systems P450 1A2 was found to catalyze M-2 and M-3 formation as well as 1′-hydroxybufuralol formation, whereas other enzymes, including P450 3A4, 2E1, and 2C9/10, showed very low or negligible activities for bufuralol hydroxylation. In microsomes from human lymphoblast cell lines P450 2D6 was found to specificically catalyze bufuralol 1′-hydroxylation. P450 1A2 in human lymphoblast cells was also found to catalyze formation of M-2, M-3, and 1′-hydroxybufuralol, although the activities observed were very low, on the basis of microsomal protein content. Because the expressed levels of P450 in human lymphoblast cell lines were reported to be ~0.03 nmol/mg of protein, the bufuralol hydroxylation activities of this P450 1A2 seem to be similar to those in the reconstituted system containing *E. coli* expressing P450 1A2.

The K_m value for bufuralol 1′-hydroxylation by recombinant P450 2D6 was estimated to be 0.018 mM; this value was much lower than the K_m value of 0.21 mM measured with recombinant P450 1A2 in reconstituted system (Fig. 14). K_m values for formation of both M-2 and M-3 by the reconstituted P450 1A2 system were found to be ~0.2 mM.

Discussion

P450 2D6 has been reported to catalyze the oxidation of a number of structurally diverse chemicals, including debrisoquine, sparteine, and bufuralol (2–4, 38). It has also been demonstrated that this enzyme shows genetic polymorphism in humans and that individuals defective in expressing the P450 2D6 gene show different responses to clinically used drugs (with respect to pharmacokinetic parameters and adverse effects), compared with the so-called extensive metabolizers, who express this protein at ordinary levels (4, 6). The possibility has also been considered that P450 2D6 genetic polymorphism may be associated with the different susceptibilities of individuals to lung cancer and Parkinson's disease, although some controversy exists (6, 39–41).

In addition to 1′-hydroxybufuralol (M-1), at least five and up to seven other products are formed from (±)-bufuralol by

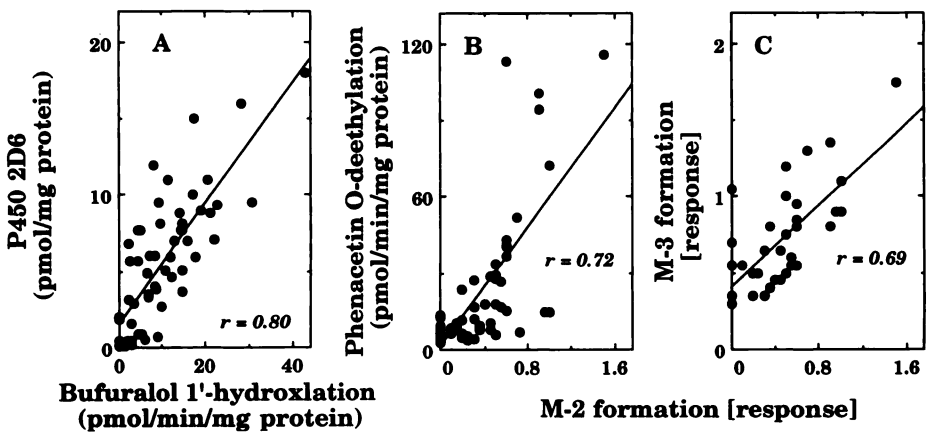


Fig. 11. Correlations between bufuralol 1′-hydroxylation and P450 2D6 contents (A), M-2 formation and phenacetin *O*-deethylation activities (B), and M-2 and M-3 formation (C) in liver microsomes prepared from 60 human liver samples. The activities for formation of the oxidative bufuralol metabolites M-2 and M-3 by human liver microsomal P450 enzymes are represented here and in Figs. 12–14 as intensities of fluorescence and UV response (per min/mg of protein or nmol of P450). Statistically significant; $p < 0.001$ in A, B, and C.

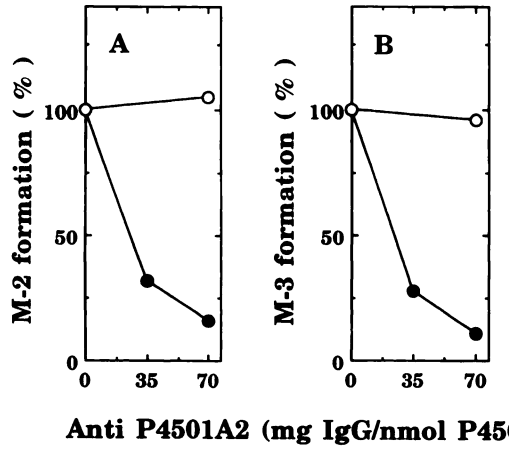


Fig. 12. Effects of anti-P450 1A2 on the formation of M-2 (A) and M-3 (B) by liver microsomes (sample HL-67). Anti-P450 1A2 IgG (●) and preimmune IgG (○) were added to the incubation mixtures at the concentrations indicated.

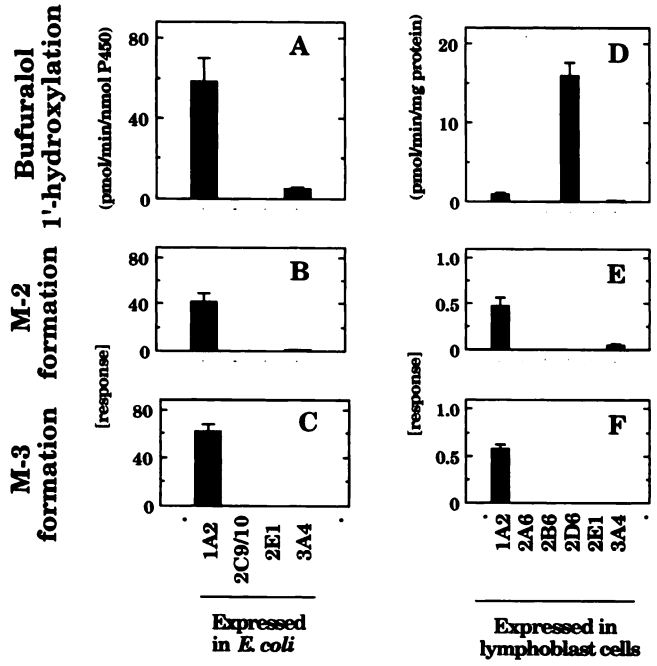


Fig. 13. Formation of 1'-hydroxybufuralol (A and D), M-2 (B and E), and M-3 (C and F) by purified P450 enzymes expressed in *E. coli* (A, B, and C) or microsomes from human lymphoblast cell lines expressing human P450 enzymes (D, E, and F).

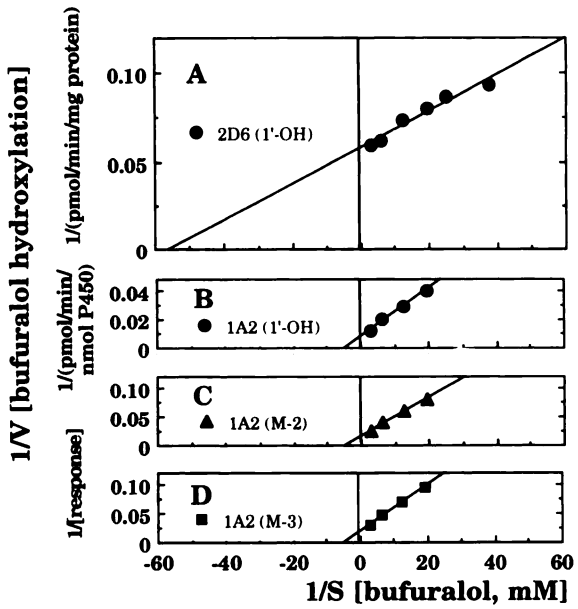


Fig. 14. Kinetic analysis of bufuralol 1'-hydroxylation and formation of M-2 and M-3 catalyzed by microsomes from human lymphoblast cells expressing P450 2D6 (A) or by purified P450 1A2 (B, C, and D) in a reconstituted monooxygenase system.

human liver microsomes and even by recombinant P450 1A2 (Fig. 2). The multiplicity of products *in vivo* was reported by Francis *et al.* (33); some of these are probably the result of multiple oxidations, and structures have been reported. We relied on the previous ^{1}H NMR assignments by Francis *et al.* (33) in discriminating the 4- and 6-hydroxy products. The 4-hydroxy product is not fluorescent; this result seems surprising but may not be in light of the report that the presence of a carbonyl function at C-1′ abolishes fluorescence (32). The ^{1}H NMR results and aromatic coupling patterns (M-2a and M-3) leave little doubt that the products must be the 4- and 6-phenols; however, the assignment of which is which is somewhat tenuous in light of our failure to obtain nuclear Overhauser effects with either compound, to establish the substitution pattern. It should also be pointed out that all of our studies have been done with racemic bufuralol as substrate. P450 2D6 is known to use both the *R*- and *S*-enantiomers as substrates but to discriminate between them (7, 42). The stereoselective hydroxylation of the two prochiral C-1′ hydrogens is also known (43–45). We have not yet explored aspects of the stereoselectivity of P450 1A2.

Francis *et al.* (33) showed that the 4- and 6-hydroxy products are excreted into human urine. Because these are associated with P450 1A2, it may be possible to utilize these reactions in noninvasive measurements, although bufuralol has not been popular as a test drug for P450 2D6. The striking lack of regioselectivity of hydroxylation for the human P450 1A enzymes is in contrast to P450 2D6, which shows nearly absolute specificity for C-1′ hydroxylation. This observation suggests that the application of pharmacophore modeling for the P450 1A2 enzyme (46) should be inherently more difficult than for P450 2D6 (47–49).

Bufuralol 1′-hydroxylation is widely used as one of the prototypical reactions catalyzed by P450 2D6 in humans and by P450 2D1 in rats. Several lines of evidence suggest that other metabolites as well as 1′-hydroxybufuralol are formed during the incubation of bufuralol with rat and human liver microsomes *in vitro* (8, 9, 45). *In vivo* experiments have also indicated the formation of several metabolites after oral administration of bufuralol to humans (32). The present results showed that a structurally unidentified metabolite, M-2, and M-3 are formed by P450 1A2 in human liver microsomes; the situation is very similar to the case with rat liver microsomes, where polycyclic hydrocarbon-inducible forms of P450 (1A1 and/or 1A2) are also involved in the formation of M-2 (9). The role of human P450 1A2 in M-2 and M-3 formation is supported in this study by the following lines of evidence: good correlation was obtained between the activities for formation of M-2 and M-3 and phenacetin *O*-deethylation, a prototypical reaction of P450 1A2 (11), in liver microsomes of 60 human samples; the formation of M-2 and M-3 by human liver microsomes was inhibited by anti-P450 1A2 and ANF; and in reconstituted

monooxygenase systems P450 1A2 had high activities for bufuralol M-2 and M-3 formation.

Complex results were obtained for the roles of human P450 enzymes in the bufuralol 1'-hydroxylation reaction. Different K_m values were obtained in seven human samples examined; low K_m values (~0.05 mM) were determined in four samples, relatively high K_m values (~0.25 mM) in two samples, and an intermediate K_m value (~0.10 mM) in one human sample. With a high substrate concentration (>0.2 mM bufuralol), at least two forms of human P450 enzymes are suggested to be involved. In human samples containing relatively high levels of P450 1A2 the bufuralol 1'-hydroxylation activities were inhibited to some extent by anti-P450 1A2 and a low concentration of ANF, whereas in P450 2D6-rich samples anti-rat P450 2D1 antibodies and quinidine were very inhibitory to the 1'-hydroxylation reaction. Using human P450 enzymes expressed in human lymphoblast cell lines or *E. coli*, we also found that P450 1A2 and P450 2D6 could catalyze bufuralol 1'-hydroxylation reactions. Gut *et al.* (7) suggested that two forms of P450, namely buf I and buf II, are involved in bufuralol 1'-hydroxylation in human liver microsomes. The former enzyme is now called P450 2D6 but the nature of the latter enzyme has not been characterized extensively. Only a single *CYP2D* subfamily gene is expressed in humans (4, 38). It is interesting to note that the K_m value of buf II for bufuralol 1'-hydroxylation (7) is very similar to the K_m value of P450 1A2 obtained in this study.

In our kinetic analysis, we could not detect biphasic parameters for bufuralol hydroxylation catalyzed by liver microsomes prepared from different human samples. Although it is not known at present why our present results showed monophasic parameters for bufuralol hydroxylation by human liver microsomes, it should be mentioned that the differences between the high and low K_m values obtained from human samples HL-50 and HL-18, respectively, were <8-fold. It should also be mentioned that Gut *et al.* (7) showed that the apparent K_m values for bufuralol hydroxylation catalyzed by liver microsomes from poor- and extensive-metabolizer phenotypes are similar to ranges obtained in this study for human samples with high and low K_m values.

However, as has been reported in several laboratories (7, 8, 50), bufuralol 1'-hydroxylation is catalyzed mainly by P450 2D6 in human liver microsomes at lower substrate concentrations. The inhibitory effects of anti-rat P450 2D1 antibodies and quinidine on the reaction catalyzed by liver microsomes support the suggestion. In addition, P450 2D6 in human lymphoblast cell lines was found to show a very low K_m value (0.018 mM), compared with K_m values of 0.21 mM for purified P450 1A2 isolated from *E. coli* membranes.

In rat liver microsomes we have obtained evidence that bufuralol 1'-hydroxylation can be catalyzed by P450 2C11 as well as P450 2D1 and P450 1A1 and 1A2 at a 0.4 mM substrate concentration (9), although P450 2D1 is the principal enzyme involved at a lower substrate concentration. We also measured the activities of human P450 enzymes belonging to the CYP2C subfamily, namely a mephenytoin 4'-hydroxylase (17) and P450 2C9/10, and found that these P450 proteins did not catalyze bufuralol 1'-hydroxylation in reconstituted monooxygenase systems.

ANF has been reported to be an inhibitor of P450 1A-catalyzed drug oxidation reactions (11, 34, 35). Our present results show that this inhibitor, when added at high concentrations, can also suppress the bufuralol 1'-hydroxylation catalyzed by human P450 2D6 but not rat P450 2D1. Other examples of species differences in the effects of the chemical inhibitors quinine and quinidine on P450 2D-supported reactions have also been reported (9, 37); quinine is very effective in inhibiting P450 2D1-dependent reactions, whereas quinidine is a specific inhibitor of P450 2D6-catalyzed drug oxidations. Although the mechanisms underlying inhibition of P450-supported monooxygenase reactions by these inhibitors may be different, our results suggest that care should be taken to study the roles of individual P450 enzymes using chemical P450 inhibitors of drug hydroxylation reactions in different species of animals.

Interindividual variations in P450-catalyzed drug oxidations have been recognized as one of the critical factors determining the different susceptibilities of individuals to drug actions and adverse reactions (51). Several factors can affect the levels of each P450 enzyme in individuals, e.g., genetics, age, gender, drug-drug interactions, and induction and/or inhibition of these P450 enzymes by environmental chemicals (1, 35, 52). As a result, the composition of P450 enzymes in liver microsomes differs among individuals and such differences may cause sometimes unexpected disorders in some human populations. The present results show that bufuralol hydroxylation activities can be affected by the ratio of P450 2D6 to P450 1A2 in liver microsomes from different humans, and they suggest the importance of detailed determination of the levels of P450 forms (for example, so-called noninvasive assays using typical substrates that are not harmful to humans) (53).

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