THE 1'-HYDROXYLATION OF RAC-BUFURALOL BY RAT BRAIN MICROSONES

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ABSTRACT:
The 1'-hydroxylation of rac-bufuralol, which is catalyzed by polymorphic CYP2D in humans, was studied in brain microsomes from male and female Wistar rats and from the female Dark Agouti rat, a model of the CYP2D6 poor metabolizer phenotype. The kinetics of the 1'-hydroxylation of bufuralol (1–1500 μM) by brain microsomes were biphasic. The activity of the high-affinity site of metabolism was consistent with Michaelis-Menten kinetics (apparent $K_m = 0.61–1.42$ μM, $V_{max}$ = 4.3–4.8 fmol/min/mg of protein), whereas the low-affinity activity was better described by a Hill function ($K_{so} = 253–258$ μM, $V_{max}$ = 817–843 fmol/min/mg of protein, $n = 1.2–1.3$). Values for kinetic constants were similar in all rat strains. Quinine was only a weak inhibitor of both the high-(apparent $K_i = 90$ μM) and low-affinity (210 μM) sites of metabolism. In contrast, the kinetics of 1'-hydroxylation of bufuralol by rat liver microsomes were best described by a two-site Michaelis-Menten function. $V_{max}$ values were 3 to 5 orders of magnitude greater compared with those for brain microsomes (male and female Wistar), and liver microsomes from female Dark Agouti rats were significantly less active than those from Wistar rats. These data, together with the known potent inhibitory effect of quinine on bufuralol 1'-hydroxylation by rat liver microsomes, indicate tissue-specific differences in the enzymology of this reaction. The role of brain CYP2D enzymes remains to be clarified.

Several forms of cytochrome P450 and their mRNA have been detected in rat and human brain (Strobel et al., 1997; McFadyen et al., 1998), albeit at concentrations of only 0.5 to 3% of those in the liver. The CYP2D subfamily contains one human gene CYP2D6 and two pseudogenes (Nelson et al., 1996). The functional expression of hepatic CYP2D6 exhibits a marked genetic polymorphism. Thus, 5 to 10% of Caucasians are homozygous for inactivating mutations of the gene (poor metabolizers) and show an impaired ability to metabolize a large number of drugs, including many that are active in the central nervous system (Tucker 1994). In the rat, five genes (CYP2D1–5) have been identified (Nelson et al., 1996) together with CYP2D18, which is probably a variant of CYP2D4 (Wan et al., 1997). Early work suggested that the enzyme deficient in the Dark Agouti (DA) rat, a model of the CYP2D6 poor metabolizer phenotype in humans, is CYP2D1 (Matsunaga et al., 1989). However, recent studies have shown that CYP2D2 and not CYP2D1, is virtually absent from the livers of female DA rats (Yamamoto et al., 1998; Schulz-Utermoehl et al., 1999).

There are limited data on the expression and catalytic activity of the CYP2D subfamily in the human brain (Bhagwat et al., 2000; Voirel et al., 2000). A cDNA fragment that is 100% homologous to the CYP2D6 gene has been identified from a human caudate cDNA library (Tyndale et al., 1991), but CYP2D6 mRNA has only been detected in the midbrain region, particularly in neurons within the substantia nigra (McFadyen et al., 1998).

Using specific anti-peptide antibodies Riedl et al. (1999) found that CYP2D is abundant in the basal ganglia of male and female rats, whereas CYP2D1 was not detected and CYP2D2 was only weakly expressed. The ability of rat brain microsomes to metabolize several substrates of hepatic CYP2D6 has been tested. Thus, Hansson et al. (1992) showed that the 10- but not the 2-hydroxylation of desipramine was catalyzed by rat brain microsomes and suggested that functional CYP2D was not present in rat brain and that 10-hydroxylation was carried out by another form of cytochrome P450. Imipramine was found to be both 10-hydroxylated (also catalyzed by human liver CYP2D6) and N-demethylated by rat brain microsomes (Sequeira and Strobel, 1996). Inhibition of 10-hydroxylation by quinidine, a potent inhibitor of human CYP2D6 activity, was presented as evidence of the involvement of CYP2D in this reaction. However, this conclusion is questionable because 1) quinidine was used at high concentrations (10 and 100 μM) that could impair its selectivity for CYP2D6, 2) quinine, not quinidine, is the more potent inhibitor of rat hepatic CYP2D (Kobayashi et al., 1989), and 3) imipramine was studied at 1 mM, a concentration at which any high-affinity CYP2D activity is likely to be saturated. Imipramine and desipramine are also N-demethylated, but not hydroxylated, by partially purified CYP2D18 (CYP2D4) (Thompson et al., 1998). Jolivalt et al. (1995) have shown that rat brain microsomes can O-demethylate dextromethorphan, also a substrate of human CYP2D6. More recently, dextromethorphan was...
found to be O-demethylated at two sites by rat brain microsomes, one of high affinity ($K_m = 2.7 \mu M$) and the other of low affinity ($K_m = 757 \mu M$) (Tyndale et al., 1999). *rac*-Bufuralol 1'-hydroxylation, another human CYP2D6-catalyzed reaction, has also been studied in rat brain but only at single and very high substrate concentrations (>200 \mu M), which are likely to saturate any high-affinity CYP2D activity (Fonne-Pfister et al., 1987; Lee and Moochhala, 1989).

We now report the characterization of the 1'-hydroxylation of bufuralol over a wide concentration range by brain microsomes from Wistar and DA rats. Because bufuralol is a very lipid-soluble compound and concentration-dependent nonspecific binding of lipid-soluble substrates to microsomal protein may influence the estimation of kinetic parameters (Obach, 1996; Tucker, 1999), its nonspecific binding to brain microsomes was also determined. Furthermore, reported instability in the rate of rat brain microsomal dextromethorphan O-demethylation after freezing and thawing of the tissue (Tyndale et al., 1999) may also affect the kinetic behavior of CYP2D substrates. Therefore, the effect of different storage conditions on the 1'-hydroxylation of bufuralol by rat brain microsomes was investigated. For the purpose of comparison, data were also generated for the 1'-hydroxylation of bufuralol by microsomes from rat liver.

Materials and Methods

Chemicals. *rac*-Bufuralol and *rac*-1'-hydroxybufuralol were gifts from Roche Products plc (Welwyn Garden City, UK). Quinine hydrochloride was purchased from Sigma Chemical Co. (Poole, Dorset, UK); glucose-6-phosphate dehydrogenase (grade II suspension), the disodium salts of NADP, and glucose 6-phosphate were purchased from Boehringer Mannheim (Leuwes, UK); and acetonitrile (HPLC grade) was purchased from Rathburn Chemicals (Walkerburn, Scotland). All other chemicals were obtained commercially and were of the highest grade available.

Source and Preparation of Brain and Liver Microsomes. Male Wistar (7–8 weeks), female Wistar (15 weeks), and female DA (15 weeks) rats were obtained from colonies bred by the University of Sheffield Field Laboratories. The animals were sacrificed by cervical dislocation followed by decapitation. The brains and livers were removed and washed in ice-cold buffer [0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 0.2 mM EDTA, 1.15% (w/v) KCl, 20% (v/v) glycerol in 0.1 mM Tris, pH 7.4] as described by Ravindranath and Anandatheerthavarada (1990). Batches of microsomes pooled from eight brains (approximately 15 g total weight) or from single rats were isolated by centrifugation. Aliquots (0.5 ml) of the resuspended microsomal fraction were frozen in liquid nitrogen and stored at −78°C for a maximum of 6 months until use. The protein concentration of microsomes was measured using the colorimetric method of Lowry et al. (1951).

Incubation Conditions. Unless otherwise stated, incubations (final volume = 200 \mu l) were carried out aerobically in 1.5-ml Eppendorf tubes at 37°C in a shaking water bath. Bufuralol (1–1500 \mu M) was preincubated, in the presence or absence of quinidine, a selective and potent inhibitor of rat hepatic CYP2D (Kobayashi et al., 1989), for 5 min in 0.2 mM potassium phosphate buffer, pH 7.4, containing 5 mM glucose 6-phosphate, 2.5 U of glucose-6-phosphate dehydrogenase, 4 mM MgCl₂, and 1.6 mM NADP. The reaction was started by the addition of microsomes (1 mg/ml brain, 0.5 mg/ml liver), and the incubations were carried out for 60 min (brain), 1 min (Wistar liver), or 5 min (DA liver). The reaction was stopped by the addition of 20 \mu l of 60% (w/v) perchloric acid, and the precipitated protein was pelleted by centrifugation (15,300 rpm for 5 min). The resultant supernatant was injected directly onto the HPLC column. The extent of inhibition of bufuralol 1'-hydroxylation by quinidine (0–500 \mu M) was determined at substrate concentrations that were approximately 0.5, 1, and 2 times the $K_m$ values for the high- and low-affinity sites of enzyme activity.

Brain Localization of Bufuralol 1'-Hydroxylase Activity. The cerebellum, olfactory lobe, and cerebral hemisphere of male Wistar rat brain were separated, and the rate of 1'-hydroxylation of bufuralol (1 \mu M and 100 \mu M) was determined in microsomes prepared from each region.

Assay of Microsomal NADPH-Reductase Activity. NADPH-reductase activity in rat brain microsomes was determined by monitoring the reduction of cytochrome c (100 nM) over time using spectrophotometry at a wavelength of 550 nm (Gibson and Skett, 1994).

Nonspecific Binding of Bufuralol to Rat Brain Microsomal Fractions. The extent of binding of bufuralol (1–1000 \mu M) to catalytically inactive brain microsomes (1 mg/ml protein) pooled from eight male Wistar rats was determined by ultrafiltration (37°C, 2000xg for 20 min) using CENTRIFREE Micropartition Devices [Millipore (UK) Ltd, Watford, UK].

HPLC Analysis of 1'-Hydroxybufuralol and Bufuralol. The HPLC comprised a model 510 pump, an RCM C18 reversed phase column (Waters, Ltd, Watford, UK) fitted with a Novapak C18 guard column (Waters), and a model FP970 fluorescence detector (Jasco, Great Dunmow, UK) with excitation and emission wavelengths set at 246 and 295 nm, respectively. The mobile phase was acetonitrile/water (13:87 v/v) containing 1% (v/v) triethylamine, adjusted to apparent pH 3 with orthophosphoric acid (70% w/v), and was delivered at a flow rate of 3 ml/min. The retention times of 1'-hydroxybufuralol and bufuralol were 4.1 and 42 min, respectively. For the analysis of samples containing quinine, the pH of the mobile phase was adjusted to 7 to allow satisfactory resolution of 1'-hydroxybufuralol and quinine. Under these conditions, the retention times of 1'-hydroxybufuralol, quinine, and bufuralol were 4.7, 22, and 60 min, respectively.

The limit of determination of 1'-hydroxybufuralol was 0.5 fmol/min/mg of microsomal protein at a baseline noise:peak height ratio of 1:4. The calibration curve for 1'-hydroxybufuralol was linear and passed through the origin ($r^2 > 0.999$). The intra-assay coefficients of variation at 20, 40, 100 fmol/sample were 3.8, 4.2, and 1.6%, respectively ($n = 10$ replicate analyses).

Bufuralol concentrations were determined using the same method as that for 1'-hydroxybufuralol except that the mobile phase was acetonitrile/water (13:87 v/v) containing 1% (v/v) triethylamine, adjusted to apparent pH 3 with orthophosphoric acid (70% w/v). Retention times were 7.3 min for bufuralol and 4.7 min for propranolol (internal standard).

Data Analysis. Data were fitted by iterative nonlinear least-squares regression (P-Pharm, Version 1.3e; SIMED, Biostatistics and Data Processing, Creteil, France), using a weighting of 1/(rate)$^2$ and based on initial estimates of $K_m$ and $V_{max}$ obtained from Eadie-Hofstee plots. All combinations of one-, two- and three-site models incorporating the Michaelis-Menten equation:

\[
y = \frac{V_{max}S}{K_m + S}
\]

(where $y$ = velocity of the reaction, $V_{max}$ = maximum velocity of the reaction, $K_m$ = substrate concentration at $y = V_{max}/2$, and $S$ = substrate concentration), and/or the Hill equation (Cornish-Bowden, 1995):

\[
y = \frac{V_{max}S^n}{K_{0.5}^n + S^n}
\]

(where $n$ is the Hill coefficient), were fitted to the data. Goodness of fit in models containing an increasing number of parameters was compared using the $F$ ratio and by applying the Akaike information criteria (Yamaoka et al., 1978).

The apparent $K_i$ for the inhibition of 1'-hydroxylation of bufuralol by quinidine in brain microsomes was determined graphically using the method of Dixon (Cornish-Bowden, 1995).

Results

Despite a relatively high lipid solubility (octanolbuffer, pH 7.4, $K_o = 46$, unpublished observation), the binding of bufuralol to catalytically inactive rat brain microsomes was low (mean = 20%). Although there was a significant correlation between bufuralol concentration (range 1–1000 \mu M) and percentage bound ($r^2 = 0.22, P < .0001$), the difference between the extent of binding at low and high concentrations was only 10% based on the line of best fit.

Brain microsomes from both Wistar and DA rats catalyzed the 1'-hydroxylation of bufuralol at rates that were linear with respect to time over 90 min of incubation at substrate concentrations of 1, 10, 50,
The effect of different experimental conditions on the 1'-hydroxylation of bufuralol by brain microsomes from male Wistar rats

Data are the mean (±S.D.) values from four replicate incubations. Experiments performed using microsomes from female Wistar and female DA rats gave similar results.

<table>
<thead>
<tr>
<th>Control</th>
<th>Percentage of Inhibition of 1'-Hydroxylation of Bufuralol (1 μM)</th>
<th>Percentage of Inhibition of 1'-Hydroxylation of Bufuralol (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−NADPH</td>
<td>94.4 ± 0.1</td>
<td>84.4 ± 0.3</td>
</tr>
<tr>
<td>−Microsomes</td>
<td>98.1 ± 0.5</td>
<td>97.2 ± 0.3</td>
</tr>
<tr>
<td>Denatured microsomes</td>
<td>96.2 ± 0.3</td>
<td>95.9 ± 0.5</td>
</tr>
<tr>
<td>+ Carbon monoxide</td>
<td>94.1 ± 0.2</td>
<td>97.8 ± 0.4</td>
</tr>
<tr>
<td>SKF-525A (1 mM)</td>
<td>84.5 ± 0</td>
<td>75.8 ± 0</td>
</tr>
</tbody>
</table>

−NADPH was not added. −microsomes, active microsomes were not added; denatured microsomes, microsomal suspension was boiled for 30 min and added in place of active microsomes; +carbon monoxide, carbon monoxide was bubbled through the incubation mixture for 1 min, and the reaction was carried out in a sealed tube; SKF-525A (1 mM), SKF-525A was coincubated with substrate.

100, and 1000 μM and up to a protein concentration of 2 mg/ml (1 μM bufuralol) (data not shown). The rate of bufuralol 1'-hydroxylation (1 μM and 1 mM) decreased by at least 90% when either the NADPH regenerating system or brain microsomes were excluded from the incubation mixture (Table 1). Greatly decreased activity was also observed in the presence of denatured microsomal protein or the cytochrome P450 inhibitor SKF-525A or when carbon monoxide was bubbled through the incubation mixture (Table 1). NADPH-cytochrome P450 reductase activity was unaffected by the presence of carbon monoxide (3.62 nmol of cytochrome c reduced/min/mg of protein before treatment; 3.50 nmol of cytochrome c reduced/min/mg of protein after treatment), confirming that the latter acts directly on cytochrome P450 holoprotein.

The localization of bufuralol 1'-hydroxylase activity in microsomes from different rat brain regions was also assessed. The appearance of 1'-hydroxybufuralol decreased in the following order: cerebellum (at 1 μM bufuralol = 2.6 ± 0.17 fmol/min/mg of protein; 200 μM bufuralol = 280 ± 90) > olfactory lobe (1 μM bufuralol = 0.56 ± 0.11; 100 μM bufuralol = 210 ± 30) > cerebral hemisphere (1 μM bufuralol = 0.40 ± 0.33; 100 μM bufuralol = 50 ± 30) [mean (± S.D.) values from six separate incubations using pooled microsomes].

To ascertain the effect of storage on bufuralol 1'-hydroxylase activity, values obtained from microsomes prepared from fresh brain and used immediately were compared with those from microsomes that had been stored at −80°C for 1 and 6 months. No loss of activity was observed at both low and high concentrations of bufuralol after 1 month, and only 15 to 20% activity was lost after 6 months [mean (± S.D.) rate of 1'-hydroxybufuralol appearance: 1 μM “fresh” = 2.62 ± 0.11 fmol/min/mg of protein from six separate incubations using pooled microsomes; 1 month = 2.58 ± 0.07; 6 months = 2.23 ± 0.22, 200 μM fresh = 598 ± 96 fmol/min/mg of protein; 1 month = 613 ± 145; 6 months = 478 ± 113].

Simple rate against substrate concentration plots for the 1'-hydroxylation of bufuralol (1–1000 μM) by brain microsomes from DA and Wistar rats were reproducibly similar in both rat strains. Those from male Wistar rat brain microsomes are shown in Fig. 1. Over the whole substrate concentration range, the plots appeared to show the hyperbolic curvature consistent with Michaelis-Menten kinetics (Fig. 1c). However, expansion of these plots revealed deviations from this model. Over the range 1 to 6 μM, the plots were characteristically hyperbolic (Fig. 1a), but between 10 and 60 μM, the increase in rate was greater than predicted, resulting in sigmoidal curvature (Fig. 1b). Deviation from simple Michaelis-Menten kinetics was even more evident on inspection of the corresponding Eadie-Hofstee plots (ν against v/S) (Fig. 2). To characterize the reaction further, the data from each rat strain were fitted by all possible combinations of one-, two-, and three-site models incorporating the Michaelis-Menten and/or Hill equations.

Quinine was a relatively weak inhibitor of bufuralol 1'-hydroxylase activity over substrate concentration ranges 2 to 6 μM and 100 to 500 μM in male Wistar rat brain microsomes, giving apparent Kᵢ values of 90 and 210 μM, respectively (each value is the mean from two individual experiments in which incubations were carried out in triplicate).

The enzyme kinetics of the 1'-hydroxylation of bufuralol by rat liver microsomes were performed under condition that were linear with respect to both time and protein concentration. Over the substrate concentration range 1 to 1000 μM, the kinetics of the reaction were best described by a two-site Michaelis-Menten function. The apparent Kᵢ values and Kᵢ values for the high an low affinity sites, respectively, were similar for both strains (Table 3). However, the apparent Vmax values and Kmax values for the high- and low-affinity sites in female DA liver microsomes were 10- and 2-fold lower, respectively, than those for female Wistar liver microsomes.

**Discussion**

The dependence of the 1'-hydroxylation of bufuralol by rat brain microsomes on the presence of NADPH and microsomes, together with its sensitivity to inhibition by carbon monoxide and SKF-525A, is consistent with catalysis by one or more cytochrome P450 enzymes. Bufuralol 1'-hydroxylase activity was highest in the cerebellum, with measurable metabolism in the olfactory lobe and cerebral hemisphere. A similar localization of enzyme activity in rat brain was observed by Tyndale et al. (1999) using dextromethorphan as the substrate.

On first inspection of the kinetic data for rat brain microsomes, the ν against ν/S plots seemed hyperbolic, which is characteristic of reactions obeying Michaelis-Menten kinetics. However, on closer scrutiny, sigmoidal behavior was apparent. This deviation from Michaelis-Menten kinetics was clearly illustrated by Eadie-Hofstee (ν versus v/S) plots of the data. If a single enzyme was involved, Eadie-Hofstee plots of data from reactions following Michaelis-Menten kinetics should be linear. If two or more enzymes with substantially differing kinetic parameters are involved, the plots will show simple negative (concave) curvature. Sigmoidal kinetics can be described by the Hill equation (Cornish-Bowden, 1995). Thus, we fitted several combinations of Hill and Michaelis-Menten equations to the data until the introduction of additional parameters gave no significant improvement of fit. The data were best described by one Michaelis-Menten function representing a high-affinity site of enzyme activity and one Hill function representing a low-affinity site. Ideally, to exclude the presence of Hill-type behavior at the high-affinity site, the substrate concentration range should have been extended to lower values. However, the sensitivity of the metabolite assay was insufficient to allow this.

Before attempting to interpret the sigmoidal behavior, it was necessary to establish that this is a property of the enzyme itself and not caused by factors relating to its environment. For example, binding of substrate to the lipid-rich microsomal membrane could influence the estimation of kinetic parameters because a lower concentration would...
be available to the enzyme. Furthermore, if such binding were substrate concentration-dependent and saturable, sigmoidal enzyme kinetics could result artifactually. Bufuralol is a highly lipid-soluble drug and might be expected to bind extensively to microsomal membranes. However, on average, only approximately 20% of added drug was found to be bound, and although there was some dependence of binding on concentration, the magnitude of the difference in binding at low and high concentrations was only approximately 10%, which is unlikely to influence interpretation of kinetic parameters to any significant extent.

Tyndale et al. (1999) reported that storage of intact rat cerebellum at $-70^\circ$C for 7 days followed by microsome preparation resulted in a 58% loss of dextromethorphan O-demethylase activity compared with that in microsomes prepared from fresh tissue. It is possible that a
are similar to those for the high-affinity site of bufuralol. Thus, larger differences in apparent $V_{\text{max}}$ values were found. Thus, after storage for 6 months, only a 15 to 20% loss of enzyme activity was seen.

Assuming that the atypical kinetics of bufuralol in rat brain are a characteristic of the enzyme(s) involved, the sigmoidal behavior could be explained by the presence of a cooperative effect. Cytochromes P450 exist in monomeric form, although they have a tendency to aggregate, which is a feature of membrane proteins (Testa, 1995). There is considerable evidence that hepatic CYP3A exhibits cooperativity (Shou et al., 1999; Witherow and Houston, 1999) and that binding of two substrates in the active site is possible (Shou et al., 1994). Recently, Savageau (1995) described a basis for sigmoidal behavior of enzymes resulting in Hill coefficients greater than unity although only a single binding site is involved in the reaction. The Hill coefficient for reactions confined to a two-dimensional membrane rather than a homogenous three-dimensional space was shown to be 1.3, whereas this value may be higher for reactions restricted to a membrane-bound enzymes in brain microsomes that are not present in liver microsomes.

The 1'-hydroxylation of bufuralol by rat liver microsomes is also associated with two or more sites of cytochrome P450 activity, but in contrast to brain sites, both appear to obey Michaelis-Menten kinetics (Table 3; Boobis et al., 1986). Values for $K_m$ in brain microsomes are similar to those for the high-affinity site of bufuralol 1'-hydroxylation in rat liver microsomes (Table 3; Boobis et al., 1986). However, values for $K_m$ were approximately 3 to 5 times higher than the corresponding $K_m$ values observed for liver microsomes. Furthermore, much larger differences in apparent $V_{\text{max}}$ values between brain and liver were found. Thus, $V_{\text{max}}$ was 5 orders of magnitude and $V_{\text{max}}$ was 3 orders of magnitude higher in liver compared with brain microsomes.

It was originally thought that, because of the similarity in the kinetic constants for bufuralol 1'-hydroxylation between brain microsomes from Wistar and DA rats, our findings were consistent with a lack of significant involvement of CYP2D2 in the high- and low-affinity bufuralol 1'-hydroxylase activities in the rat brain. However, during the course of this work, Riedl et al. (1999) demonstrated that although the DA rat lacks hepatic CYP2D2, its expression in the midbrain is similar to that of Wistar rats. Thus, the DA rat may not be an appropriate model for probing rat CYP2D2 activity. However, the relatively weak inhibition of bufuralol 1'-hydroxylase by quinine provides evidence against a significant contribution from CYP2D2, although this is based on the assumption that the behavior of the liver and brain forms of CYP2D2 are the same. Clearly, our data do not exclude a role for the other forms of CYP2D. CYP2D5 is the most abundant isofrom of the CYP2D family in rat brain (Riedl et al., 1999), but there are no published data on its catalytic activity with respect to substrates of CYP2D6. Heterologously expressed CYPs 2D1 to 2D4 all display some bufuralol 1'-hydroxylase activity but were less active than CYP2D6 (Wan et al., 1997). Fonse-Pflister et al. (1987) suggested that cumene hydroperoxide-mediated (+)-bufuralol 1'-hydroxylase activity observed in rat brain microsomes was mediated by CYP2D1 (now shown to be CYP2D2). The latter conclusion was based on significant inhibition of bufuralol oxidation by human antibodies (anti-LKM-1). However, the selectivity of anti-LKM-1 antibodies with respect to individual members of the CYP2D subfamily has not been evaluated. Furthermore, it has been reported that anti-LKM-1 antibodies are also capable of inhibiting the activity of cytochromes P450 from other families (Waxman et al., 1988). Thus, although the enzymology of bufuralol 1'-hydroxylation is clearly different in rat brain compared with liver microsomes, the role, if any, of the individual members of the CYP2D subfamily in this reaction needs further delineation. In contrast to the present findings on bufuralol 1'-hydroxylation, Tyndale et al. (1999) concluded that the O-demethylation of dextromethorphan by rat brain microsomes was catalyzed by CYP2D1. This interpretation was based partly on observations of differential inhibition of enzyme activity by quinine and quinidine. However, as in our studies, the effect of quinine was relatively weak compared with its potency in rat liver microsomes (Kobayashi et al., 1989). Furthermore, the difference in inhibitory potency between quinidine and quidine found by Tyndale et al. (1999) was more than an order of magnitude less than that reported for rat liver CYP2D (Kobayashi et al., 1989). More recent work by Voiron et al. (2000) also showed weak inhibition of rat brain microsomal dextromethorphan O-demethylation by quinidine. However, a poly-

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (pmol/min/mg)</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male Wistar</td>
<td>1.42 ± 0.02</td>
<td>4.8 ± 0.03</td>
<td>253.0 ± 0.5</td>
<td>1.3 ± 0.001</td>
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<tr>
<td>Female Wistar</td>
<td>0.91 ± 0.27</td>
<td>4.3 ± 0.02</td>
<td>258.4 ± 0.4</td>
<td>1.2 ± 0.001</td>
</tr>
<tr>
<td>Female DA</td>
<td>0.61 ± 0.15</td>
<td>4.5 ± 0.02</td>
<td>258.4 ± 0.4</td>
<td>1.2 ± 0.0008</td>
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<thead>
<tr>
<th>Rat Strain</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (pmol/min/mg)</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male Wistar</td>
<td>0.92 ± 0.20</td>
<td>1041 ± 11</td>
<td>53 ± 21</td>
<td>1140 ± 15</td>
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<tr>
<td>Female Wistar</td>
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<td>1345 ± 4</td>
<td>84 ± 20</td>
<td>1548 ± 7</td>
</tr>
<tr>
<td>Female DA</td>
<td>0.91 ± 0.26</td>
<td>117 ± 2</td>
<td>36 ± 17</td>
<td>405 ± 7</td>
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clonal antibody against CYP2D6 caused a 60% decrease in the rate of this reaction in rat brain that is consistent with the involvement of a CYP2D enzyme.

In summary, at least two cytochrome P450 enzymes seem to catalyze the 1'-hydroxylation of bufuralol in rat brain microsomes, but further work is needed to identify the enzymes involved. In addition, enzyme activity associated with the low-affinity site shows marked sigmoidal behavior, which may be explained by cooperativity or by spatial restriction on metabolism.

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