ABSTRACT:

We tested the ability of human liver microsomes (HLMs) and recombinant human cytochrome P450 (CYP or P450) isoforms to catalyze the N-demethylation of nirvanol-free (S)-mephenytoin ([S]-MP) in vitro. In mixed HLMs, the kinetics of (S)-MP N-demethylation suggested two contributing activities. A high-affinity/low-capacity component exhibited a $K_M$ of 174.1 $\mu$M and a $V_{max}$ of 170.5 pmol/mg protein/min, whereas a low-affinity/high-capacity component exhibited a $K_M$ of 1911 $\mu$M and a $V_{max}$ of 3984 pmol/mg protein/min. The activity of the high-affinity component was completely abolished by sulfaphenazole, with little effect on the low-affinity component. Of the recombinant P450 isoforms tested, only CYP2B6 and CYP2C9 formed nirvanol from (S)-MP. The $K_M$ value (150 ± 42 $\mu$M) derived for recombinant CYP2C9 was close to that obtained for the high-affinity/low-capacity component in mixed HLMs ($K_M = 174.1 \mu$M). The predicted contribution of this activity at concentrations (1–25 $\mu$M) achieved after a single 100-mg dose of racemic MP is approximately 30% of the rate of nirvanol formation. At concentrations of >1000 $\mu$M, we estimate that >90% of the rate can be explained by the low-affinity activity (CYP2B6). Therefore, the N-demethylation of (S)-MP to nirvanol may be a useful means of probing the activity of CYP2B6 in vivo when concentrations of >1000 $\mu$M are used, but it is unlikely to be a suitable phenotyping tool for this isoform in vivo, where concentrations of >1000 $\mu$M are rarely encountered.

Mephenytoin (MP) is a 3-methyl-5-phenyl-5-ethylhydantoin an antiepileptic agent that is also a well-recognized probe drug for the CYP2C19 metabolic polymorphism (Küpfer et al., 1984). This chiral drug undergoes stereoselective metabolism (Küpfer et al., 1981) in which the $S$-$(+)$-enantiomer is hydroxylated at the 4’-position but also undergoes N-demethylation to an active metabolite (Troupin et al., 1976), i.e. nirvanol (5-phenyl-5-ethylhydantoin). The $R$-$(–)$-enantiomer is primarily N-demethylated (Küpfer et al., 1981).

In humans, the primary route of (S)-MP metabolism after a single 100-mg dose of the racemate is to (S)-4’-OH-MP (Küpfer et al., 1984). This reaction is catalyzed by a genetically polymorphic P450 isoform, CYP2C19 (Wrighton et al., 1993; Goldstein et al., 1994), and forms the basis for the most common tests used to determine the CYP2C19 phenotype (Wedlund et al., 1984). During chronic therapeutic use of the drug at doses of 100–600 mg/day, N-demethylation of (S)-MP to nirvanol is predominant (Küpfer et al., 1984; Troupin et al., 1979). The enzymes that catalyze N-demethylation of (S)-MP to nirvanol in vivo have not been identified. Using HLMs in vitro, this reaction has been shown to be catalyzed by CYP2B6 at substrate concentrations (0.2–3 mM) (Heyn et al., 1996) that may be relevant to total serum MP concentrations (40 $\mu$g/ml or 183 $\mu$M) observed after chronic clinical dosing (Küpfer et al., 1981).

A growing list of substrates for recombinant CYP2B6 have been identified in vitro, using several indirect methods of characterization (literature cited in Ekins et al., 1997). There is evidence that CYP2B6 is expressed in HLMs, with wide interindividual variability that could be the result of genetic polymorphism or environmental exposure (Code et al., 1997). However, investigations of hepatic micromosal CYP2B6 have generally been limited because of the lack of specific substrate probes and specific chemical inhibitors or immunoinhibitors (Ekins et al., 1997; Guo et al., 1997). Consequently, little is presently known about its actual role in xenobiotic oxidative metabolism. Recently, (S)-MP N-demethylation to nirvanol was recommended and used as a probe for CYP2B6 in vitro (Heyn et al., 1996), but the concentrations used were 20–150 times the peak concentrations (13 $\mu$M) of (S)-MP achieved after the standard dose of 100 mg used to determine the metabolic phenotype of CYP2C19, even in poor metabolizers with respect to this isoform (Küpfer et al., 1984). It follows that, if CYP2B6 is the only enzyme catalyzing this reaction in vivo, then this substrate could be used to simultaneously probe CYP2C19 and CYP2B6 in vitro and probably also in vivo. In this study, we determined the kinetics of N-demethylation of (S)-MP across a wide range of substrate concentrations and identified the specific P450 isoforms involved in this reaction, paying particular attention to the concentrations that appear relevant after the single 100-mg dose used to determine metabolic phenotypes.

Materials and Methods

Chemicals and Reagents. Chlorzoxazone, dextromethorphan hydrobromide, phenacetin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, EDTA, and tolbutamide were purchased from Sigma Chemical Co. (St.
Louis, MO). (S)-MP (99.3% pure), nirvanol, and sulfaphenazole were purchased from Ultrafine Chemicals (Manchester, UK).

HLMs. The preparation and metabolic characteristics of the HLMs were as described previously (Harris et al., 1994). The microsomes were resuspended to a protein concentration of 5–12 mg/ml in reaction buffer (0.1 M sodium/potassium phosphate, 1.0 mM EDTA, 5.0 mM MgCl2, pH 7.4) and were stored at −80°C until used. Protein concentrations were determined using the method described by Pollard et al. (1978).

Preparation of Nirvanol-Free (S)-MP. Contaminant nirvanol in (S)-MP was eliminated by differential collection of fractions from HPLC. (S)-MP was dissolved in 50% methanol in water to a concentration of 10 mg/ml, and 100 µl were injected directly onto a Microsorb C18 (5 µm, 150 mm × 4.6 mm) analytical column (Rainin Instrument Co., Woburn, MA). The flow rate of the mobile phase (30% methanol in water) was 1.0 ml/min, and the eluant was monitored using a Waters (Milford, MA) model 490 UV detector, at a wavelength of 211 nm. Collected solutions were evaporated to dryness by speed vacuum, and the samples were weighed. Extraction efficiency was 80–90%, and reination of the purified (S)-MP (10 mg/ml, 100 µl) showed that the amount of nirvanol remaining was below the lower limit of quantification of the assay (50 ng/ml, <0.0005%). Stock solutions of nirvanol-free (S)-MP were prepared by dissolution in 100% methanol (10 mg/ml for assays with HLMs and 9 mg/ml for assays with recombinant P450 isoforms) and were then sequentially diluted with water to prepare the concentrations used. The highest final concentrations of methanol in incubations were 0.44% and 0.46%, respectively.

Incubation Conditions and Enzyme Assays. The formation of nirvanol from (S)-MP was tested by incubating appropriate concentrations of nirvanol-free (S)-MP with HLMs, using incubation conditions that were linear for time from (S)-MP was tested by incubating appropriate concentrations of nirvanol (0.5–200 µM). Reactions were stopped by the addition of 100 µl of cold acetonitrile. After addition of 100 µl of cold acetonitrile, the aqueous layer was extracted by the addition of 3 ml of methylene chloride, and the organic layer was removed, centrifuged at 2000 rpm for 5 min in a Beckman J-6M centrifuge (JS4.0 rotor), and then dried in a speed vacuum. Dried pellets were reconstituted with 250 µl of mobile phase, and 100 µl were injected into the HPLC apparatus. Rates of production of metabolite were quantified by using the ratio of the AUC of the metabolite to the AUC of the internal standard phenytoin. Instruments used for HPLC were controlled by a Waters Millennium 2010 chromatography manager and included a Waters model 600 HPLC pump, Waters model 717 autosampler, and Waters model 490 UV detector. Standard curves for nirvanol were linear in two concentration ranges (0.1–20 µg/ml and 6.3–200 ng/ml).

cDNA-Expressed Human P450 Isoforms. cDNA-expressed human P450 isoforms (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) were purchased from Gentest Corp. (Woburn, MA). Protein concentrations and P450 contents were as supplied by the manufacturer. The suspensions of cDNA-expressed HLMs were stored at −80°C and thawed at 37°C before incubation. Screening for nirvanol formation by these isoforms was performed using a (S)-MP concentration of 200 µM and a protein concentration of 0.76 mg/ml, except in the assay using CYP3A4 (1.4 mg/ml). The experiments using recombinant CYP2C9 to examine the kinetics of nirvanol formation were performed using 2 mg/ml cDNA-expressed material and a (S)-MP concentration range of 0.5–100 µM. After preincubation (5 min) of a mixture containing the substrate (with or without inhibitor) and an NADPH-generating system, reactions were initiated by the addition of cDNA-expressed P450 isoforms and were incubated for 120 min. The procedures for extraction and measurement of metabolite were the same as described for the assay with HLMs. For the recombinant CYP1A2 and CYP2E1 assays, any alcohol component in the substrate was eliminated by evaporation to dryness and reconstitution of the substrate in 0.1 M phosphate buffer before the start of the incubation. Each assay included a positive control with a documented substrate for each pathway, i.e., phenacitin O-deethylation for CYP1A2, tolbutamide 4-methoxyhydroxylation for CYP2C9, (S)-MP 4-hydroxylation for CYP2C19, dextromethorphan O-demethylation and N-demethylation for CYP2D6 and CYP3A, respectively, and chlorozoxazone 6-hydroxylation for CYP2E1. Each positive control was analyzed using a method developed for the detection of the relevant metabolite (Ko et al., 1997).

Data Analysis. Graphical analyses were performed using the Excel (Microsoft Corp., Redmond, WA) graphics package. Initial estimates acquired by linear regression of unweighted data in Eadie-Hofstee plots were used to determine the apparent Km and Vmax values through a nonlinear regression analysis (WINNONLIN version 1.5; Scientific Consulting Inc., Lexington, KY), using the following two-site binding equation:

\[
V = \frac{V_{\text{max},1}}{[S] + K_{\text{M},1}} + \frac{V_{\text{max},2}}{[S] + K_{\text{M},2}}
\]

where \(K_{\text{M},1}\) and \(V_{\text{max},1}\) represent the high-affinity/low-capacity site and \(K_{\text{M},2}\) and \(V_{\text{max},2}\) represent the low-affinity/high-capacity site. Iterative calculation was performed with 1/w2 weighting.

Results and Discussion

We characterized the kinetics of nirvanol formation from (S)-MP by incubating nirvanol-free (S)-MP (0.5–200 µM) with pooled HLMs (HL2, HL8, and HL9). The results depicted as a Michaelis-Menten plot in fig. 1A demonstrate that N-demethylation of (S)-MP to nirvanol is saturable. Our results do not concur with earlier reports that suggested that this pathway is nonsaturable at the substrate concentrations used (Meier et al., 1985; Jurima et al., 1985; Hall et al., 1987). In contrast to reports that the N-demethylation of (S)-MP is catalyzed by a single enzyme, visual inspection of Eadie-Hofstee plots of our data showed biphasic kinetic behavior (fig. 1B), suggesting the involvement of at least two enzymatic activities. These activities were best described by a two-site model with high-affinity/low-capacity (\(K_{\text{M},1}\) and \(V_{\text{max},1}\)) and low-affinity/high-capacity (\(K_{\text{M},2}\) and \(V_{\text{max},2}\)) components. The kinetic parameters derived using a nonlinear regression analysis for two sites are shown in table 1. Given the wide interindividual variability of the nirvanol formation rate (Heyn et al., 1996), it is possible that the kinetic parameters we document here for the low-affinity component represent CYP2B6-mediated nirvanol formation (Heyn et al., 1996), whereas the high-affinity component represents activity that has not been described before. To test the variability of the low- and high-affinity components of the reaction, we repeated the experiment using pooled HLMs from other liver preparations (HL10, HL16, and HL17). The data in all cases were best characterized by a two-site enzymatic activity. The initial kinetic parameter estimates derived from these data were 208.6 and 1060 µM for \(K_{\text{M},1}\) and \(K_{\text{M},2}\) and 46.9 and 471 pmol/min/mg protein for \(V_{\text{max},1}\) and \(V_{\text{max},2}\), respectively. Because of the large (>20-fold) \(V_{\text{max}}\) difference between the two activities and because the formation of nirvanol might be low in certain liver preparations, the high-affinity component would not be detected if experiments were conducted at high concentrations (in the millimolar range) and might be obscured by trace contamination of (S)-MP with nirvanol. This high-affinity component might be important in clinical settings, because of the relatively low in vivo plasma concentrations of (S)-MP (<15 µM) (Troupin et al., 1979) and because of the fact that nirvanol, not 4-OH-MP, is the major metabolite when MP is used chronically (Küper et al., 1984; Wedlund et al., 1984).

To determine which P450 isoforms are involved in the N-demeth-
Nirvanol-free (S)-MP was incubated for 60 min with pooled HLMs (HL2, HL8, and HL9). Data are presented as a Michaelis-Menten plot (A) and an Eadie-Hofstee plot (B). Dotted lines, high- and low-affinity components calculated by nonlinear regression.

**TABLE 1**

Estimated kinetic parameters for the formation of nirvanol from nirvanol-free (S)-MP (0.5–200 μM), in the presence or absence of sulfaphenazole (25 μM), in pooled HLMs (HL2, HL8, and HL9).

<table>
<thead>
<tr>
<th>Incubation with</th>
<th>Kinetics of Nirvanol Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_M,1 (μM)</td>
</tr>
<tr>
<td>(S)-MP alone</td>
<td>174.1</td>
</tr>
<tr>
<td>(S)-MP + sulfaphenazole</td>
<td>2,706</td>
</tr>
</tbody>
</table>

K_M,1 and V_max,1 refer to Michaelis-Menten parameters for the high-affinity component, whereas K_M,2 and V_max,2 refer to the low-affinity component. Values are means of estimates from nonlinear least-squares regression analysis, performed using WINNONLIN (see Materials and Methods).

**TABLE 2**

Rate of nirvanol formation from (S)-MP (200 μM) by pooled HLMs and recombinant P450 isoforms.

<table>
<thead>
<tr>
<th>Rate of Nirvanol Formation</th>
<th>pmol/mg protein/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled HLMs</td>
<td>43.6</td>
</tr>
<tr>
<td>Recombinant P450 isoforms</td>
<td></td>
</tr>
<tr>
<td>1A2</td>
<td>NA</td>
</tr>
<tr>
<td>2B6</td>
<td>43.6</td>
</tr>
<tr>
<td>2C9</td>
<td>12.0</td>
</tr>
<tr>
<td>2C19</td>
<td>NA</td>
</tr>
<tr>
<td>2D6</td>
<td>NA</td>
</tr>
<tr>
<td>2E1</td>
<td>NA</td>
</tr>
<tr>
<td>3A4</td>
<td>NA</td>
</tr>
</tbody>
</table>

*NA, not active.*

**FIG 1.** Nirvanol formation from (S)-MP.

Recombinant CYP2C9 in N-demethylation of (S)-MP. We found that the reaction catalyzed by recombinant CYP2C9 showed a low V_max (17.4 ± 1.8 pmol/mg protein/min), whereas the K_M value (150 ± 42 μM) was close to the average K_M,1 value (174.1 μM) obtained with mixed HLMs (table 1). Our data suggest that CYP2C9 may be an important catalyst of (S)-MP N-demethylation to nirvanol at clinical plasma concentrations resulting from the single 100-mg dose of racemic MP [containing 50 mg of (S)-MP] routinely used to determine the metabolic phenotype of CYP2C19. At these concentrations (1–25 μM), the predicted contribution of the high-affinity component would be 30% (calculated using the Michaelis-Menten equation for a two-site model). It is possible that the relevant in vivo concentrations at the metabolic site in the liver are higher and that the contribution of CYP2C9 is lower than this estimate. Because we do not have reliable means of estimating the substrate concentrations at the active site in
the liver, this question might be addressed by clinical studies that
document the effects of a CYP2C9 inhibitor, such as fluconazole
(Black et al., 1996; Mitra et al., 1996), on the partial metabolic
clearance of (S)-MP to (S)-nirvanol.

The high-affinity/low-capacity component of (S)-MP N-demethyl-
ation (CYP2C9) appears to contribute significantly at low concentra-
tions that may be present after a single dose used for metabolic
phenotyping or during chronic therapy. Given the wide interindividual
variability in the activities of CYP2C9 and CYP2B6, it is likely that
the relative contributions of these enzymes to nirvanol formation
would vary with the relative expression of each isoform in different
livers. Under our experimental conditions with HLMs, approximately
90% of the rate of nirvanol formation can be explained by the
low-affinity/high-capacity component. Although we recognize the
need for highly specific substrate probes for CYP2B6 activity, we
agree with Heyn et al. (1996) that (S)-MP at high concentrations (>1
mM) is useful as an in vitro probe for this isoform. However, because
the low-affinity \$K_M\$ value (1911 \$\mu\text{M}\$) we found was 11 times greater
than the \$K_M\$ value for the higher affinity component (174.1 \$\mu\text{M}\$),
N-demethylation of (S)-MP is not likely to be a useful phenotyping
tool for CYP2B6 in vivo, where concentrations of >1 mM are rarely
encountered.

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