QUANTITATIVE CONTRIBUTION OF CYP2D6 AND CYP3A TO OXYCODONE METABOLISM IN HUMAN LIVER AND INTESTINAL MICROSONES

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ABSTRACT:

Oxycodone undergoes N-demethylation to noroxycodone and O-demethylation to oxymorphone. The cytochrome P450 (P450) isoforms capable of mediating the oxidation of oxycodone to oxymorphone and noroxycodone were identified using a panel of recombinant human P450s. CYP3A4 and CYP3A5 displayed the highest activity for oxycodone N-demethylation; intrinsic clearance for CYP3A5 was slightly higher than that for CYP3A4. CYP2D6 had the highest activity for O-demethylation. Multienzyme, Michaelis-Menten kinetics were observed for both oxidative reactions in microsomes prepared from five human livers. Inhibition with ketoconazole showed that CYP3A is the high affinity enzyme for oxycodone N-demethylation; ketoconazole inhibited >90% of noroxycodone formation at low substrate concentrations. CYP3A-mediated noroxycodone formation exhibited a mean $K_m$ of 600 ± 119 $\mu$M and a $V_{max}$ that ranged from 716 to 14523 pmol/mg/min.

Contribution from the low affinity enzyme(s) did not exceed 8% of total intrinsic clearance for N-demethylation. Quinidine inhibition showed that CYP2D6 is the high affinity enzyme for O-demethylation with a mean $K_i$ of 130 ± 33 $\mu$M and a $V_{max}$ that ranged from 89 to 356 pmol/mg/min. Activity of the low affinity enzyme(s) accounted for 10 to 26% of total intrinsic clearance for O-demethylation. On average, the total intrinsic clearance for noroxycodone formation was 8 times greater than that for oxymorphone formation across the five liver microsomal preparations (10.5 $\mu$mol/min/gm versus 1.5 $\mu$mol/min/gm). Experiments with human intestinal mucosal microsomes indicated lower N-demethylation activity (20-50%) compared with liver microsomes and negligible O-demethylation activity, which predict a minimal contribution of intestinal mucosa in the first-pass oxidative metabolism of oxycodone.

Oxycodone (4,5-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one; 14-dihydroxycodine) is an opioid analgesic widely used for the treatment of postoperative pain (Nuutinen et al., 1986; Silvasti et al., 1998; Curtis et al., 1999) and pain associated with cancer (De Conno et al., 1991; Glare and Walsh, 1993; Parris et al., 1998). It has also been suggested for the management of nonmalignant cancer (De Conno et al., 1991; Glare and Walsh, 1993; Parris et al., 1998). Oxycodone is extensively metabolized; only 10% of dose is excreted unchanged in urine (Poyhia et al., 1991; Kirvela et al., 1996). The known metabolic scheme of oxycodone is presented in Fig. 1. Oxymorphone, a 3-O-demethylation metabolite of oxycodone, is a potent opioid that has a 3 to 5 times higher $\mu$-opioid receptor affinity than morphine (Childers et al., 1979; Chen et al., 1991). Oral oxymorphone is 10-fold more potent than oral morphine based on dose (Melmon et al., 2000).

Cytochrome P450 (P450) enzyme CYP2D6 is known to catalyze the O-demethylation of 3-methoxy-17-methyl morphinans, e.g., codeine, to morphine (Dayer et al., 1988) and dextromethorphan to dextromethorphan (Kronbach et al., 1987; Dayer et al., 1989). Morphine derived from the oxidation of codeine has been shown to account for most, if not all, of the analgesic activity of codeine (Caraco et al., 1996a; Poulsen et al., 1996). Until recently, oxycodone O-demethylation to oxymorphone was thought to be a CYP2D6-mediated bioactivation process, similar to codeine. Otton et al. (1993) demonstrated that CYP2D6 is the principal O-demethylase; oxymorphone formation in human liver microsomes was much lower in CYP2D6 poor metabolizers, as compared with extensive metabolizers, and inhibited by quinidine, a selective CYP2D6. Also, Somogyi (1999) has reported that oxymorphone formation in human liver microsomes was inhibited by anti-CYP2D6. However, blockade of oxymorphone formation by quinine has been shown not to decrease the antinociceptive effect of oxycodone in rats (Cleary et al., 1994). Inhibition of CYP2D6 by quinidine did not attenuate the opioid-induced side effects of oxycodone in human volunteers (Kaiko et al., 1996; Heiskanen et al., 1998).

Oxycodone also undergoes N-demethylation at the 17-position to noroxycodone (Weinstein and Gaylord, 1979; Somogyi, 1999). CYP3A4 is known to catalyze the N-demethylation of codeine (Gor- skii et al., 1994) and dextromethorphan (Caraco et al., 1996b), but the role of this enzyme in the N-demethylation of oxycodone has not been established. Leow and Smith (1994) reported that noroxycodone exhibited modest analgesic potency after i.c.v. administration in the rat.
Noroxycodone or its metabolites may be a source of active metabolites that play a role in the analgesia of oxycodone. Other metabolic pathways involving 6-keto reduction and conjugation of oxycodone have been identified in animals (Ishida et al., 1979; 1982) but have not been assessed in humans.

We have conducted in vitro metabolic studies to identify the specific P450s involved in the oxidation of oxycodone and to assess their respective contribution toward the intrinsic metabolic clearance of each pathway. The ability of a panel of recombinant human P450s to O- and N-demethylate oxycodone was examined. This was followed by studies in human liver and intestinal microsomes to 1) fully characterize the Michaelis-Menten kinetics of noroxycodone and oxymorphone formation, and 2) assess the contribution of the identified P450 isofor(s) through incubation studies with P450-specific inhibitors.

Materials and Methods

Materials. Oxycodone hydrochloride, quinidine, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Ketaconazole was obtained from Sigma-Aldrich (Kansas City, KS). The manufacturers provided the microsomal protein concentrations used in the human liver microsomal suspensions at protein concentrations used in the human liver microsomes were purchased from Xenotech, LLC and assayed for microsomal protein, 0.1 to 800 μg protein, and 1 mM NADPH in 100 mM, pH 7.4 phosphate buffer. The substrate was added to the microsomal suspension, and the oxidative reactions were started with the addition of NADPH after a 5-min pre-equilibration period. All incubations were performed in duplicate, in a 37°C water bath-shaker over time intervals varying from 0 to 30 min. The inhibitors, ketocazole and quinidine, were dissolved in an 80:20 (v/v) mix of methanol:water. An aliquot of the inhibitor was placed in the incubation tubes and the solution was evaporated to dryness under N₂ for 15 min. Addition of other components of the incubation mixture followed. Metabolic reactions were terminated by protein precipitation with the addition of 1 ml of ice-cold acetonitrile. The tubes were immediately vortexed for 2 to 3 s and immersed in ice. At a later time, internal standards d₃-NOC and d₃-OM were added at 20 ng each. Samples were then spun in a microcentrifuge at 16,000 g for 5 min to pellet the protein. The supernatant was collected and dried under N₂ in a fresh tube. The samples were reconstituted in 150 μl of mobile phase, of which 1 to 5 μl was injected onto the liquid chromatograph-mass spectrometer for metabolite analysis.

Metabolite Analysis. Oxycodone metabolites in human liver microsomes were analyzed by liquid chromatography-mass spectrometry (LC-MS). Chromatographic separation of the analytes was achieved with a 5-μm, 2.1 mm × 150 mm Zorbax SSB C18 column (Agilent Technologies, Palo Alto, CA). The mobile phase consisted of an 88:12 (v/v) mix of 10 mM, pH 4 potassium phosphate buffer and methanol. The mass spectrometer was operated in the atmospheric pressure ionization electrospray mode with positive polarity. The m/z 340 [M + K]⁺ ion was monitored for oxycodone and the m/z 302 [M + H⁺]⁺ ion for noroxycodone; m/z 343 [M + K]⁺ and m/z 305 [M + H⁺]⁺ were monitored for the respective deuterated internal standard analogs. The molecular ion for oxycodone was not routinely monitored.

Calibration standards and quality controls were prepared in heat-inactivated human liver microsomal suspensions at protein concentrations used in the kinetic experiments (0.2 mg/ml). Peak area ratios of oxycodone to oxymorphone-d₃ were linear over the range of 0.1 to 150 ng when the [M + K]⁺ ion was monitored. For noroxycodone, the linear range was 0.25 to 1000 ng when the [M + H⁺]⁺ ion was monitored. Limits of quantification were 0.25 ng per 0.5 ml (0.2 ng/ml) of microsomal incubates for oxymorphone, and 0.25 ng per 0.5 ml (0.5 ng/ml) of incubate for noroxycodone. The intraday (within-batch) accuracy and precision of the LC-MS assay was determined by replicate assay of spiked microsomal samples (n = 15) at three different concentration levels corresponding to low, intermediate, and high regions of the calibration range; i.e., 6.6, 75, or 125 ng of oxymorphone and 26.6, 250, or 750 ng of noroxycodone. Interday quality control data were obtained by assaying the same spiked samples in duplicate over 5 consecutive days. The interday and intraday mean values were not significantly different from each other. Their coefficients of variation were well less than 10%.

Initial analysis revealed a trace, containing presence of oxymorphone and noroxycodone in the commercial supply of oxycodone. The percentage of each contaminating compound was determined and applied as a correction factor for both the calibration standards and the incubate samples. For the calibration standards, the correction being constant was 0.1% for noroxycodone and 0.01% oxymorphone. For the incubate samples, the amount of metabolite formed. The maximum correction was 15% of the observed amount of noroxycodone formed, and 1 to 2% of the amount of oxymorphone formed.

Optimization of Incubation Condition. To establish the optimal condition for initial velocity measurement, we determined the linearity of noroxycodone and oxymorphone formation with respect to time (0.5–40 min) and microsomal protein concentration (0.1–5 mg/ml) at various substrate concentrations.
Additionally, a mass-balance study was performed to examine whether the loss of oxycodone during the incubation period could be accounted for by the formation of noroxycodone and noroxycodone; i.e., to rule out the presence of any unrecognized, competing metabolic processes. Oxycodone at 2 μM was incubated in triplicates over 0, 30, 60, 90, and 120 min in Xenotech liver microsomes. Oxycodone depletion was measured as the difference between the oxycodone concentration observed at the incubation stop-times and the initial oxycodone concentration.

cDNA-Expressed P450s. To identify which human P450 isoforms mediate the formation of noroxycodone and oxymorphone, oxycodone was incubated with a panel of human lymphoblastoid microsomes expressing individual human P450 enzymes. Incubations were performed with microsomes expressing 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. No oxycodone turnover was observed in control incubations performed with microsomes transfected with expression vector alone. Reactions were carried out at 5, 15, and 150 μM oxycodone, using the procedure outlined in the preceding section. The concentration of microsomal protein used in these experiments was as recommended by the manufacturer. Additional P450 metabolic screening was conducted with noroxycodone and oxymorphone as substrates to examine formation of the common di-demethylated secondary metabolite noroxycodone.

To further define the saturation kinetics of oxymorphone and noroxycodone formation from oxycodone, as well as noroxycodone from either oxymorphone or noroxycodone, incubations were performed over the substrate range of 1 to 750 μM in Supersomes expressing CYP3A4, 3A5, and 2D6*1, either with or without supplementation of cytochrome b5 (i.e., at 3:1 molar ratio of cytochrome b5 to P450).

Noroxycodone and Oxymorphone Formation Kinetics. Initial velocities of noroxycodone and oxymorphone formation were determined at 15 concentrations of oxycodone that ranged from 0.1 to 800 μM. Parallel sets of incubations at 10, 30, 100, 300, 650, or 800 μM oxycodone were performed in the presence of 20, 60, or 200 nM quinidine (CYP2D6 inhibitor) or ketoconazole (CYP3A inhibitor). The chosen inhibitor concentrations ranged from 1 to 10 times the known Ki, whereas the substrate concentrations ranged from below to above the oxycodone Km (Otton et al., 1993; Gibbs et al., 1999).

For each preparation of human liver microsomes, formation velocity data for noroxycodone and oxymorphone in the presence and absence of an inhibitor were fitted to either a one- (eq. 1) or two-enzyme model (eqs. 2 and 3). The two-enzyme models consisted of either two saturable (Michaelis-Menten) components (eq. 2) or a single saturable (MM) component and a second, linear component (eq. 3).

\[ v = \frac{V_{\text{max} 1} \cdot S}{K_{\text{m1}} + S} \]  
(1)

\[ v = \frac{V_{\text{max} 1} \cdot S}{K_{\text{m1}} + S} + \frac{V_{\text{max} 2} \cdot S}{K_{\text{m2}} + S} \]  
(2)

\[ v = \frac{V_{\text{max} 1} \cdot S}{K_{\text{m1}} + S} + E_0 \cdot S \]  
(3)

\( v \) represents the metabolite formation velocity, \( K_{\text{m1}} \) the Michaelis-Menten constant of the substrate for the high affinity enzyme, \( V_{\text{max} 1} \) the maximum formation velocity for the high affinity enzyme, \( S \) the substrate concentration, and \( E_0 \) the \( v/K \) ratio for the high or low affinity enzyme.

Three simple inhibition models, i.e., competitive, noncompetitive, and uncompetitive inhibition, were considered for the effects of quinidine and ketoconazole as defined in the equations below (Segel, 1975), with \( K_i \) as the dissociation constant of the inhibitor-enzyme complex and \( I \) the inhibitor concentration. Metabolite formation velocity data from control incubations and incubations with inhibitor were fitted simultaneously to the model equations.

Competitive Inhibition \( K_{\text{cat, i}} = K_{\text{cat}} \cdot \left(1 + \frac{I}{K_i}\right) \)  
(4)

Noncompetitive Inhibition \( V_{\text{max} i} = \frac{V_{\text{max} i, 0}}{1 + \frac{I}{K_i}} \)  
(5)

Ketoconazole and quinidine were assumed to inhibit the high affinity component of each oxidative reaction. Nonlinear regression fit of data to the model equations was performed using the numeric module of SAAM II (Version 1.1; University of Washington, Seattle, WA). A fractional standard deviation error model (10%) was applied to the fits based on known analytical errors.

The choice of enzyme models was judged by the F-test ratio (unweighted residual sum of squares), the Akaike information criterion value, plots of residuals, visual inspection of the model fit, and the 95% confidence intervals of the parameter estimates.

Oxycodone oxidation by microsomes isolated from the intestinal mucosa was examined at a single substrate concentration (10 μM). A parallel set of incubations was performed with fixed single inhibitor concentrations of quinidine and ketoconazole (100 nM) to assess the involvement of CYP3A and 2D6 in the intestinal metabolism of oxycodone.

Microsomal Protein Binding. To determine the extent to which oxycodone binds to human liver microsomes, we performed equilibrium dialysis studies using a Spectra/Por Equilibrium Dialyzer (Spectrum Laboratories Inc., Rancho Dominguez, CA) equipped with microcells (0.5 ml/cell). Regenerated cellulose membrane discs with a 12- to 14-kDa cutoff (Spectrum Laboratories Inc.) were rinsed in distilled water and soaked in 100 mM, pH 7.4 phosphate buffer prior to use. Microsomal suspensions (0.2 mg/ml of protein) spiked with oxycodone were dialyzed against in 100 mM, pH 7.4 phosphate buffer. The experiments were conducted at two substrate concentrations, 7 and 70 μM, over 3 and 6 h. The microsomal free fraction was expressed as the ratio of buffer side (free) concentration divided by the protein side (total) concentration. The dialysates were assayed according to the LC-MS method described above.

Results

Linearity of Metabolite Formation and Mass Balance. To establish the appropriate microsomal incubation conditions for initial velocity measurements, linearity of metabolite formation was observed over a 40-min incubation period and over a protein concentration range of 0.1 to 5 mg/ml, independent of substrate concentration. Negligible loss of oxycodone or formation of metabolites was detected in control incubations without the addition of NADPH.

A mass-balance experiment was performed to determine whether the N- and O- demethylation reactions quantitatively account for the metabolic loss of oxycodone. Substrate consumption was <5% over the first 30 min, increasing to 20% by 120 min. Figure 3 shows that loss of oxycodone over the 2-h incubation was reasonably accounted for by the formation of oxymorphone and noroxycodone, and no apparent competing oxidative processes were observed. Although the secondary metabolite noroxycodone was not quantitated, it may account for the small differences between parent loss and primary metabolite formation observed at 90 and 120 min.

cDNA-Expressed Human P450s. A set of lymphoblastoid microsomes expressing individual P450 enzyme was used to examine which human drug-metabolizing P450 isoforms are capable of transforming oxycodone to noroxycodone and oxymorphone. Figure 2 shows the oxidative activity of individual P450s in forming noroxycodone and oxymorphone at 15, 150, and 500 μM oxycodone. The formation velocity is normalized by P450 content of each microsomal preparation. CYP3A4 showed the highest specific activity for conversion of oxymorphone at 15, 150, and 500 μM, and 150 μM oxycodone was incubated in triplicates over 0, 30, 60, 90, and 120 min in Xenotech liver microsomes. Oxycodone depletion was measured as the difference between the oxycodone concentration observed at the incubation stop-times and the initial oxycodone concentration.
CYP2D6 catalyzed the convergent, secondary metabolism of oxymorphone and noroxycodone to noroxymorphone. CYP2D6 showed barely detectable activities at the highest substrate concentration in microsomes from all five human donor livers, which reflect the consistent achievement of noroxycodone formation at the highest ketoconazole concentration of 200 \(\mu M\) in microsomal preparations, which illustrate different kinetic behavior between liver microsomes. To better resolve the multienzyme kinetics and ascertain which kinetic component reflects CYP3A-mediated oxidation, we extended our kinetic experiments to include incubations with ketoconazole, a CYP3A-selective inhibitor.

Figure 4 shows representative saturation profiles of noroxycodone formation and the effect of ketoconazole obtained in two microsomal preparations, which illustrate different kinetic behavior between livers. HLM 141 data exhibited multienzyme kinetics, whereas HLM 148 data followed uni-enzyme kinetics. The distinction was most evident when their Dixon plots were compared; i.e., curvilinear Dixon plot for HLM 141 in contrast to a clearly linear Dixon plot for HLM 148. Greater than 90% inhibition of \(N\)-demethylation activity was consistently achieved at the highest ketoconazole concentration of 200 \(\mu M\) in microsomes from all five human donor livers, which reflect the dominant role of CYP3A in the formation of noroxycodone. This is consistent with the earlier experiments with cDNA-expressed P450s.

Noroxycodone formation velocity data from individual human liver microsomes were fit to either a single-enzyme Michaelis-Menten equation (eq. 1) or a two-enzyme model consisting of a saturable, Michaelis-Menten component and a linear, low affinity term (eq. 3). Attempts to fit the data to more complex models, the two-enzyme Michaelis-Menten model (eq. 2) or three-enzyme models, either failed to converge or yielded poorer fits. The single-enzyme Michaelis-Menten model best described noroxycodone formation in Xenotech, HLM 148, and HLM 153 microsomes. The two-enzyme model (eq. 3) was best for HLM 141 and HLM 146. Estimates of noroxycodone kinetic parameters for each of the five human liver microsomal preparations are presented in Table 1. The competitive model was consistently superior to the noncompetitive and uncompetitive models in describing the inhibition kinetics of ketoconazole for all five liver preparations.

Data modeling confirmed CYP3A as the high affinity enzyme for noroxycodone formation. As indicated above, a low affinity, non-CYP3A-related component was identifiable in HLM 141 and HLM 146. Even in those two livers, the contribution of low affinity enzyme(s) in the overall formation velocity at low substrate concentration \(<K_{m1}\) was minor; intrinsic clearance due to the low affinity component \(E_2\) accounted for merely 7 to 8% of the total intrinsic clearance \(E_1 + E_2\) of the \(N\)-demethylation pathway.
A mean \( K_{\text{m1}} \) estimate of \( 600 \pm 119 \) \( \mu M \) was observed for CYP3A-mediated oxycodone \( N \)-demethylation in the five liver microsomal preparations (see Table 1), along with a large, 2.5-fold interliver variation in the \( V_{\text{max1}} \). To investigate the reason for the large interliver variability in CYP3A-mediated noroxycodone formation, the content of CYP3A4 and CYP3A5 (picomoles of P450 per milligram of protein) was determined by Western blotting. The CYP3A4 and CYP3A5 content varied some-what in parallel, the ratio of their content differed remarkably between microsomal preparations. HLM 146 contained little CYP3A5. HLM

<table>
<thead>
<tr>
<th>Liver Microsomes</th>
<th>( K_{\text{m1}} ) ( \mu M )</th>
<th>( V_{\text{max1}} ) pmol/min</th>
<th>( E_i ) ( \mu M \cdot \text{mg}^{-1} \cdot \text{min}^{-1} )</th>
<th>( E_2 ) ( \mu M \cdot \text{mg}^{-1} \cdot \text{min}^{-1} )</th>
<th>( K_i ) nM</th>
<th>CYP3A4 Content( ^{b} ) pmol/mg</th>
<th>CYP3A5 Content( ^{b} ) pmol/mg</th>
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<tr>
<td>Xenotech</td>
<td>524</td>
<td>6071</td>
<td>11.6</td>
<td>17.9</td>
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<td>(4525–7618)</td>
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<td></td>
<td></td>
<td>(14.8–21.1)</td>
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<tr>
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<td>804</td>
<td>14,523</td>
<td>18.1</td>
<td>1.41</td>
<td>25.1</td>
<td>70.6</td>
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<tr>
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<td>(9536–19,510)</td>
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<td>(0.02–2.6)</td>
<td>(17.3–33.0)</td>
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<tr>
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<td>716</td>
<td>1.2</td>
<td>0.09</td>
<td>15.5</td>
<td>10</td>
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<td>(325–1107)</td>
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<td>(6.5–28.5)</td>
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Mean ± S.D. 600 ± 119 6577 ± 5070 10.2 ± 6.3 0.7 ± 0.9 20.8 ± 5.5

\( ^{a} \text{N.D., not detected.} \)
\( ^{b} \text{Data were previously reported by Lin et al. (2002).} \)

The mean \( K_{\text{m1}} \) of 600 ± 119 \( \mu M \) was observed for CYP3A-mediated oxycodone \( N \)-demethylation in the five liver microsomal preparations (see Table 1), along with a large, 2.5-fold interliver variation in the \( V_{\text{max1}} \). To investigate the reason for the large interliver variability in CYP3A-mediated noroxycodone formation, the content of CYP3A4 and CYP3A5 (picomoles of P450 per milligram of protein) was determined by Western blotting. The CYP3A4 and CYP3A5 content varied some-what in parallel, the ratio of their content differed remarkably between microsomal preparations. HLM 146 contained little CYP3A5. HLM 141, on the other hand, displayed unusually high CYP3A5 content, 3-fold higher than its CYP3A4 content. The other two livers, HLM 148 and 153, contained CYP3A5 at levels lower than that of CYP3A4. The rank order of \( V_{\text{max1}} \) and \( E_i \) estimates corresponded well with that of total CYP3A content, i.e., CYP3A4 plus CYP3A5.

The competitive \( K_i \) estimates of ketoconazole for CYP3A ranged between 15 and 28 nM, in agreement with published data (Gibbs et al., 1999). The presence of ketoconazole had no significant effect on oxymorphone formation, which is consistent with the absence of \( O \)-demethylation when oxycodone was incubated with cDNA-expressed CYP3A4.

In human intestinal microsomes, noroxycodone formation rate per milligram of protein at 10 \( \mu M \) oxycodone was 2- to 5-fold lower than in hepatic microsomes (data not shown). Noroxycodone formation was inhibited by 60 to 80\% upon addition of 100 nM ketoconazole.

Oxymorphone Formation in Human Liver Microsomes. Initial analysis of the oxymorphone formation kinetics also suggested the involvement of at least two enzymes. To investigate whether CYP2D6 is the high affinity \( O \)-demethylase, the effect of quinidine was examined. Figure 5 shows representative saturation plots of oxymorphone formation in two human liver microsomes. All of the formation velocity versus substrate concentration plots failed to display a defi-nite saturation plateau. For all five microsomal preparations, data were well described by a two-enzyme model consisting of a Michaelis-Menten and a linear term (eq. 3), along with competitive inhibition by quinidine (eq. 4). The model parameter estimates for the five human liver microsomal preparations are listed in Table 2. The mean \( K_{\text{m1}} \) estimate for CYP2D6 was 130 ± 33 \( \mu M \); \( O \)-demethylation \( V_{\text{max1}} \) varied by 4-fold across the five liver preparations. The low affinity, non-CYP2D6 component \( (E_2) \) accounted for 11\% to 26\% of total intrinsic clearance \( (E_i + E_2) \). The significant presence of a non-CYP2D6 component in the formation of oxymorphone is also reflected in the pronounced curvilinear Dixon plots as shown in Fig. 5 (right panel).

The mean \( K_i \) of quinidine on CYP2D6-mediated \( O \)-demethylation of oxycodone was 13 ± 8 nM, which is within the range of reported \( K_i \) values of quinidine on CYP2D6-mediated oxidation of other substrates.

In human intestinal microsomes, oxymorphone formation rate at 10 \( \mu M \) oxycodone was at least 10- to 20-fold lower than in hepatic microsomes per milligram of protein; at 100 nM quinidine, no oxymorphone formation was detected.

Microsomal Protein Binding. Nonspecific binding of drug sub-strates in microsomal incubates restricts their access to the enzymes. Hence, correction of in vitro intrinsic metabolic clearance \( (V_{\text{max1}} / K_{\text{m}}) \) for nonspecific binding is recommended, particularly when the in vitro clearance estimates are scaled to predict in vivo organ clearances (Obach, 1999). To determine whether oxycodone binds to the micro-somal matrix, equilibrium dialysis experiments were performed on two of the microsomal preparations, HLM 148 and 153. These microsomal preparations exhibited negligible oxycodone binding at substrate concentrations of 7 and 70 \( \mu M \), which is within the range of reported \( K_i \) values of quinidine on CYP2D6-mediated oxidation of other substrates.

In human intestinal microsomes, oxymorphone formation rate at 10 \( \mu M \) oxycodone was at least 10- to 20-fold lower than in hepatic microsomes per milligram of protein; at 100 nM quinidine, no oxymorphone formation was detected.

Kinetic Studies with Supersome CYP3A4, CYP3A5, and CYP2D6. To compare the activities of CYP3A4 and CYP3A5 in noroxycodone formation, saturation kinetics of oxycodone \( N \)-demeth-ylation were determined using Supersomes recombinant human...
CYP3A4 and CYP3A5. The kinetic experiments were performed with and without cytochrome b₅ supplementation (3:1 cytochrome b₅ to P450) to examine the effect of this electron donor on the CYP3A-mediated reaction.

N-Demethylation of oxycodone by CYP3A4 exhibited a typical hyperbolic profile, whereas CYP3A5-catalyzed noroxycodone formation did not reach apparent saturation up to a substrate concentration of 750 μM. Kinetic modeling using a uni-enzyme Michaelis-Menten model (eq. 1) yielded estimates for CYP3A4 noroxycodone formation with and without cytochrome b₅ supplementation (Table 3). Upon supplementation of cytochrome b₅, increases in Kₘ (2-fold) and k₉ (5-fold) were observed; intrinsic clearance increased by 2.6-fold. The Kₘ for CYP3A4 N-demethylation in the presence of cytochrome b₅ supplementation was closer to the observed Kₘ for CYP3A3-mediated oxycodone N-demethylation in liver microsomes.

Similar to CYP3A4, addition of cytochrome b₅ increased oxycodone CYP3A5 intrinsic clearance by more than 2-fold. The apparent lack of saturation over the substrate concentration range suggests that Kₘ for CYP3A5-mediated oxycodone N-demethylation is in the millimolar range. The intrinsic clearance estimated from the slope of the linear portion of the velocity versus substrate concentration plot (<200 μM) exceeded that of CYP3A4 by 33% and 22% in the respective absence and presence of cytochrome b₅. There was a reasonably good prediction of Vₘₐₓ for microsomes from each of the human livers based on the cytochrome b₅-supplemented Supersome CYP3A4 and CYP3A5 turnover data and the specific content of CYP3A4 and CYP3A5.

O-Demethylation of oxycodone and noroxycodone by CYP2D6*1 Supersomes was examined. This preparation of recombinant CYP2D6 contained coexpressed cytochrome b₅ and cytochrome P450 reductase. Hyperbolic saturation plots were observed for both oxycodone and noroxycodone (Fig. 6). The Kₘ for Supersome CYP2D6-mediated oxycodone O-demethylation was on average 3-fold lower than the Kₘ in human liver microsomes. CYP2D6 Supersomes efficiently catalyzed the O-demethylation of noroxycodone to noroxymorphone. Kₘ for CYP2D6-mediated noroxycodone O-demethylation was 2-fold lower, and Vₘₐₓ was 20% higher than 2D6-mediated oxycodone O-demethylation; hence, intrinsic clearance for O-demethylation of noroxycodone was 2.5-fold higher than that of oxycodone (Table 3).

**Discussion**

The role of P450 enzymes in the metabolism of weak oral opioids has attracted significant attention since the early 1990s when it was recognized that CYP2D6 catalyzes O-demethylation of codeine to morphine and is a major determinant of its analgesic efficacy. However, follow-up studies with other 3- and 17-methylated morphine analogs failed to show a similar pharmacodynamic importance of CYP2D6-mediated O-demethylation as with codeine (vide infra). Attention has recently shifted to the other P450 enzymes involved in the metabolism of opioids with respect to their potential role in opioid pharmacokinetics, vis-à-vis active metabolite formation.

We undertook the present study to identify the major P450 enzymes responsible for oxycodone N-demethylation to noroxycodone and O-demethylation to oxymorphone, and to assess their relative contribution to the oxidative metabolism of oxycodone using human liver and intestinal microsomes.

N-Demethylation of oxycodone to noroxycodone represents the predominant oxidative pathway in human liver microsomes and is almost exclusively (>90%) catalyzed by CYP3A. For the minor O-demethylation pathway, CYP2D6 accounted for 79 to 90% of total intrinsic clearance for oxymorphone formation in the five preparations of human liver microsomes. In the present panel of human liver microsomes, the intrinsic clearance for CYP3A3-mediated N-demethylation was 2 to 14 times the intrinsic clearance for the parallel CYP2D6-mediated O-demethylation; mean intrinsic clearance for noroxycodone formation was 10.5 μmol/min/mg versus 1.5 μmol/min/mg for oxymorphone formation. This is consistent with the data from experiments performed with cDNA-expressed enzymes, which showed a 6-fold higher specific activity of CYP3A4-mediated N-demethylation versus CYP2D6-mediated O-demethylation. Scale-up of our in vitro, microsomal intrinsic clearance values (excluding the data from CYP3A3-deficient HLM 146) predicted an in vivo hepatic clearance that was in excellent agreement with the i.v. oxycodone clearance (i.e., within 80–112% of the reported value of 0.78 l/min) (Pyhia et al., 1991). In the human intestinal microsomes, oxycodone oxidation occurred at a much lower rate than that in the human liver microsomes. Assuming a 2- to 5-fold lower N-demethylation intrinsic clearance in the intestinal mucosa, the first-pass intestinal extraction is predicted to be ~2%, negligible compared with a predicted liver extraction of 23%.

A high interliver variation in microsomal N-demethylation of oxycodone was noted. Genetic polymorphism in the expression of CYP3A5 has recently been recognized as a major contributing factor to the well recognized intersubject variability of CYP3A activity in vivo (Lamba et al., 2002; Lin et al., 2002). This led us to investigate the comparative oxidative activity of recombinant CYP3A4 and CYP3A5 toward oxycodone. The intrinsic clearance of CYP3A5 was

**Table 2**

<table>
<thead>
<tr>
<th>Liver Microsomes</th>
<th>Kₘ (μM)</th>
<th>Vₘₐₓ (μmol/mg/min)</th>
<th>Eₙ (μl/mg/min)</th>
<th>Eₜ (μl/mg/min)</th>
<th>k₉ (nM)</th>
<th>CYP2D6 Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xenotech</td>
<td>113</td>
<td>(63–163)</td>
<td>92</td>
<td>(55–128)</td>
<td>0.81</td>
<td>0.17</td>
</tr>
<tr>
<td>HLM141</td>
<td>149</td>
<td>(102–210)</td>
<td>115</td>
<td>(237–475)</td>
<td>2.39</td>
<td>0.71</td>
</tr>
<tr>
<td>HLM146</td>
<td>178</td>
<td>(98–258)</td>
<td>64</td>
<td>(63–166)</td>
<td>0.64</td>
<td>0.09</td>
</tr>
<tr>
<td>HLM148</td>
<td>114</td>
<td>(59–179)</td>
<td>156</td>
<td>(101–211)</td>
<td>1.37</td>
<td>0.15</td>
</tr>
<tr>
<td>HLM153</td>
<td>98</td>
<td>(67–130)</td>
<td>89</td>
<td>(64–114)</td>
<td>0.91</td>
<td>0.16</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>130 ± 33</td>
<td>161 ± 112</td>
<td>1.2 ± 0.7</td>
<td>0.21 ± 0.22</td>
<td>13.4 ± 7.7</td>
<td></td>
</tr>
</tbody>
</table>

* Eₙ = Vₘₐₓ/Kₘₐₓ.

Data were previously reported by Madani et al. (1999).
OXYCODONE OXIDATION IN VITRO

SOMOGYI (1999). The abstract reported a mean 

TABLE 3

Estimates of Michaelis-Menten parameters for oxycodone metabolite formation in Gentest Supersomes containing recombinant CYP3A4, CYP3A5, and CYP2D6

<table>
<thead>
<tr>
<th>Reaction</th>
<th>P450</th>
<th>K_m (μM)</th>
<th>k_cat (1/min)</th>
<th>CL_int (μl/min/pmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxycodone→Noroxycodone</td>
<td>3A4</td>
<td>377</td>
<td>4.7</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>3A4 + b_5</td>
<td>(235–519)</td>
<td>(3.78–5.57)</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>3A4 + b_5</td>
<td>(595–744)</td>
<td>(20.28–23.14)</td>
<td></td>
</tr>
<tr>
<td>Oxycodone→Noroxycodone^a</td>
<td>3A5</td>
<td>3A5 + b_5</td>
<td>39.8</td>
<td>11.4</td>
</tr>
<tr>
<td>Oxycodone→Oxymorphone</td>
<td>2D6*1</td>
<td>20.5</td>
<td>14.9</td>
<td>0.727</td>
</tr>
<tr>
<td>Noroxycodone→Noroxymorphone</td>
<td>2D6*1</td>
<td>(15.2–25.9)</td>
<td>(14.1–15.7)</td>
<td></td>
</tr>
</tbody>
</table>

^a CYP3A5-mediated formation of noroxycodone did not reach apparent saturation within the substrate concentration used.

The upper panels show the formation kinetics of noroxycodone, oxymorphone, and noroxymorphone in Gentest Supersomes. The lower panels show the formation kinetics of oxymorphone from oxycodone (left) and noroxymorphone from noroxycodone (right) by Gentest Supersomes CYP2D6.

comparable with that of CYP3A4. CYP3A5 expression could explain the between-liver variability in oxycodone N-demethylation. Indeed, there was a rank order correlation of N-demethylation intrinsic clearance with the sum of CYP3A4 and CYP3A5 content. It is interesting to note that HLM 141, which has the highest content of CYP3A5 in our human liver bank, exhibited the highest intrinsic clearance for oxycodone N-demethylation.

The present K_m estimates for noroxycodone and oxymorphine formation from oxycodone are lower than values reported in a recent abstract by Somogyi (1999). The abstract reported a mean K_m for oxycodone N-demethylation of 1.6 ± 0.2 mM, compared with our 600 ± 119 μM; V_max values were similar to those from this study. It was mentioned that ketoconazole and CYP3A4 antibody were able to inhibit noroxycodone formation completely, which is consistent with our present finding with ketoconazole. The abstract further reported a mean K_m of 262 ± 81 μM for oxycodone O-demethylation by liver microsomes from four "extensive metabolizers" of CYP2D6, which is 2-fold higher than our K_m estimate of 130 ± 33 μM. The K_m for microsomes from one “poor metabolizer” (PM) was even higher at 924 μM. V_max was reported at 135 ± 22 pmol/mg/min for the extensive metabolizer microsomes and 49 pmol/mg/min for the PM microsomal (Somogyi, 1999); the former value is reasonably similar to our present estimate of 161 ± 112 pmol/min. None of the human livers in our study carry the common CYP2D6 PM genotype.

The differences in K_m estimates between the two studies may be related to the difficulty in resolving the multi-enzyme kinetics of P450-mediated oxycodone oxidation. Initial enzyme kinetic modeling of velocity data, in the absence of inhibitors, failed to conclusively discriminate between uni- and multi-enzyme models. We refined our experimental approach to include incubations with CYP3A- and CYP2D6-specific inhibitors, i.e., ketoconazole and quinidine. This design allowed a defining assignment of the high affinity catalytic component of oxycodone N-demethylation to CYP3A and that of O-demethylation to CYP2D6. Although the inclusion of the inhibition data requires estimation of an additional K_i term, the model parameters were estimated with greater precision due to the additional information.

The experiment with lymphoblast microsomes expressing individual P450s suggested that, aside from CYP3A and CYP2D6, other P450 enzymes (e.g., CYP2C19) may be involved in the formation of both noroxycodone and oxymorphone. Quantitative contribution of these other isoenzymes appears to be minor, at least at their constitutive level of expression.

Our in vitro findings are consistent with available in vivo data from pharmacokinetic studies in human volunteers and patients. Very low (<2 ng/ml) circulating concentrations of oxycodone have been observed following a single oral dose of oxycodone; the AUC ratios oxymorphone to parent drug were <0.01. On the other hand, plasma noroxycodone concentrations were reported to be significantly higher and comparable with the parent drug; noroxycodone to parent AUC ratios were slightly above unity (Poyhia et al., 1991, 1992; Kaiko et al., 1996; Heiskanen et al., 1998). A low presence of circulating oxymorphone could either be explained by its limited formation or efficient clearance (i.e., 3-O-glucuronidation). On the other hand, the high circulating concentration of noroxycodone could be attributed to either extensive N-demethylation of oxycodone or a slow clearance of noroxycodone. The present microsomal data point to a very limited extent of O-demethylation, while clearly establishing N-demethylation as a major primary pathway in oxycodone metabolism.

Studies to date suggest a minor role of oxymorphone in the pharmacodynamics of oxycodone. Cleary et al. (1994) examined the effect of quinine pretreatment (potent inhibition of CYP2D1) on the antinociceptive effect of oxycodone in Sprague-Dawley rats. No attenuation of...
antinociception was observed with quinine pretreatment. Heiskanen et al. (1999) investigated the effects of CYP2D6 inhibition by quinine on the side-effects of slow release oxycodone in 10 healthy human volunteers, who were extensive metabolizers of CYP2D6. There was no change in the subjective or psychomotor effects of oxycodone following cotreatment with quinine as compared with placebo, despite a dramatic decrease in plasma oxymorphone due to inhibition of CYP2D6.

These available oxycodone pharmacodynamic data do not follow the recognized importance of CYP2D6-mediated activation in codeine pharmacology but are consistent with findings from other structurally related 6-keto oral opioids in terms of the limited impact of CYP2D6-generated metabolites on their opioid-related effects. CYP2D6 inhibition studies with hydrocodeine or dihydrocodeine have all failed to demonstrate any significant impact on subjective opioid response in human subjects (Kaplan et al., 1997) or antinociception in rats (Tomkins et al., 1997; Wilder-Smith et al., 1998).

The prominence of N-demethylation in oxycodone metabolism raises the possibility that this pathway may be a source of active metabolites that contribute to the pharmacodynamics of oxycodone. As mentioned earlier, noroxycodone is an active opioid, albeit modest in antinociceptive effect and side effects. Further in vivo studies will be required to ascertain whether noroxycodone, noroxymorphone, and any other metabolites derived from the N-demethylation pathway have a significant role in the pharmacodynamics of oxycodone.

In summary, our study offers a full elucidation of oxycodone oxidation by human liver P450 enzymes. We identified CYP3A4 to be the principal N-demethylase and CYP2D6 the major O-demethylase of oxycodone in human liver microsomes. N-Demethylation is the major oxidative pathway; hence, CYP3A expression and activity are expected to be a major determinant of oxycodone clearance in vivo, e.g., pharmacologic expression of CYP3A5. Our finding of a limited extent of CYP2D6-mediated O-demethylation is consistent with the previously reported low circulating concentration of oxymorphone following oral oxycodone administration in human subjects, which explains its apparent lack of contribution to the pharmacodynamics of oxycodone. Finally, the active secondary oxidative metabolite, noroxymorphone, is shown to be an O-demethylation product of noroxycodone and may have an important role in the pharmacodynamics of oxycodone.

References


