ROLE OF HUMAN LIVER CYTOCHROME P4503A IN THE METABOLISM OF ETORICOXIB, A NOVEL CYCLOOXYGENASE-2 SELECTIVE INHIBITOR

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(Received October 19, 2000; accepted February 9, 2001)

This paper is available online at http://dmd.aspetjournals.org

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of pain, inflammation, and fever (Donnelly and Hawkey, 1997; Jouzeau et al., 1997). The mechanism of action of many of these drugs (e.g., meloxicam, lornoxicam, tenoxicam, and indomethacin) is thought to involve the inhibition of COX, a hemeprotein that has been shown to convert arachidonic acid to proinflammatory prostaglandins and their subsequent metabolic products (Wu, 1998). COX behaves as an endoperoxide synthetase, catalyzing the formation of the cyclic endoperoxide prostaglandin G2 from the unesterified precursor fatty acid, and as a peroxidase that converts prostaglandin G2 to prostaglandin H2. Prostaglandin H2 is then converted to other products (e.g., prostaglandin I2 and thromboxane A2) via the action of prostacyclin synthetase and thromboxane synthetase.

COX has been shown to exist in two forms (COX-1 and COX-2). COX-1 is thought to carry out “housekeeping” functions (e.g., cytoprotection of the gastric mucosa and platelet aggregation) and catalyzes the production of prostaglandins under normal physiological conditions. By comparison, COX-2 is normally undetectable in most tissues and is inducible by cytokines, endotoxins, and tumor promoters (Donnelly and Hawkey, 1997; Jouzeau et al., 1997). Since the products of COX-1 are cytoprotective, selective inhibition of COX-2 is anticipated to reduce inflammation without the gastrointestinal side effects characteristic of NSAIDs currently in use (Donnelly and Hawkey, 1997; Jouzeau et al., 1997; Riendeau et al., 1997; Wu, 1998). Therefore, much attention has been focused on the design of potent and selective COX-2 inhibitors (Leblanc et al., 1995, 1999; Penning et al., 1997; Janusz et al., 1998). One such example is etoricoxib (Fig. 1), which is under investigation for the treatment of osteoarthritis and rheumatoid arthritis. The drug has been shown to be a potent and selective inhibitor of COX-2 in vitro and exhibits pharmacological activity in vivo (Friesen et al., 1998; Riendeau et al., 2000). In an earlier study, the in vitro metabolites of etoricoxib [5-chloro-3-(4-methanesulfonylphenyl)-6'-methyl-[2,3’]-bipyridinyl] were characterized, and preliminary studies with recombinant P450s suggested that multiple P450s were involved in the oxidative metabolism of the drug (Chauret et al., 2001). Although etoricoxib is a low clearance compound (0.8 ml/min/kg), with high oral bioavailability (>80%), it has been shown to undergo extensive biotransformation (>90% of the dose) to metabolites that are detected in urine and feces (R. Halpin and N. Agrawal, unpublished observations). Because it is likely that etoricoxib will be administered with other drugs in a clinical setting,
we sought to study the in vitro NADPH-dependent metabolism of [\(^{14}\)C]etoricoxib in native human liver microsomes and to identify the P450 forms involved in its metabolism. We also wanted to determine the relevance of the in vitro data to the situation in vivo by determining the metabolite profile of etoricoxib in human urine.

**Materials and Methods**

**Chemicals.** Unlabeled etoricoxib and [\(^{14}\)C]etoricoxib (labeled at the methyl group of the methanesulfonylphenyl substituent; 28 \(\mu\)Ci/mg, radiochemical purity \(>98\%\)) were synthesized at Merck Research Laboratories (West Point, PA, and Rahway, NJ). The 1'-N'-oxide (M1), 6'-hydroxymethyl (M2), and 6'-carboxy (M3) metabolites of etoricoxib were provided by R. Friesen (Merck-Froest Center for Therapeutic Research, QC, Canada). Ketaconazole, sulfaphenazole, furafylline (Research Biologics, Inc., Natick, MA), and (+) -mephenytoin (Saltford Ultrafine Chemicals and Research, Manchester, UK) were purchased from the sources indicated. Omeprazole sulfone and fluvoxamine were obtained from the Merck compound repository (West Point, PA). All other commercially available reagents and solvents were of either analytical or HPLC grade. Insect cell microsomes (“Supersomes”) containing various individual cDNA-expressed P450 proteins and NADPH-P450 reductase were obtained from GENTEST Corp. (Woburn, MA). Microsomes prepared from cells containing the selectable plasmid vector without cDNA insert, and the supernatant was decanted into a clean tube. After evaporation to dryness under nitrogen at 37°C, the dried residue was reconstituted in 0.2 ml of acetonitrile (30%, v/v). An aliquot of sample (0.1 ml) was analyzed by HPLC with radiometric detection.

**Kinetic Analysis.** Estimates of apparent \(K_m\) and \(V_{max}\) were obtained by fitting the untransformed data to a one- or two-enzyme model (PCNONLIN version 4.0; Statistical Consultants, Inc., Lexington, KY) using 16 different concentrations of etoricoxib (Rodrigues et al., 1996). Initial kinetic parameter estimates were obtained using EnzymeKinetics (Macintosh version 1.3; Trinity Software, Compton, NH). Data were also analyzed by linear transformation (Eadie-Hofstee plot) to confirm a single \(K_m\) model. The in vitro M2 formation \(\text{CL}_{\text{int}, \text{in vitro}} (V_{\text{max}}/K_m)\) was scaled with respect to the yield of microsomal protein (45 mg/g liver) and liver weight (26 g liver/kg b.wt.). Protein binding in microsomes \((f_u, \text{inc})\) was determined at two concentrations of [\(^{14}\)C]etoricoxib (5 and 200 \(\mu\)M) with an ultrafiltration technique using the Centriffue Micropartition System (Amicon, Beverly, MA). Drug was incubated with human liver microsomes (1.0 mg protein/ml) at 37°C in 0.1 M potassium phosphate buffer (pH 7.4). At both concentrations of drug, protein binding was low (<12% bound), and \(\text{CL}_{\text{int, in vitro}}\) was corrected with respect to free fraction \((f_u, \text{inc} = 0.88).\) Thus, the predicted (free) intrinsic clearance of etoricoxib in vivo \((\text{CL}_{\text{int, in vivo (pred)}}; \text{ml/min/kg of b.wt.)}) was calculated using the following relationship (Obach, 1996; Obach et al., 1997):

\[
\text{CL}_{\text{int, in vivo (pred)}} = \text{CL}_{\text{int, in vitro}} (1/f_u, \text{inc}) \cdot 45 \cdot 26 \cdot (1/1000).
\]

**Measurement of P450 Form Selective Activities.** Microsomal samples (Xenotech, LLC) were prepared for incubation and analysis as described previously. The rate of M2 formation, at each concentration of etoricoxib (5 and 200 \(\mu\)M), was correlated with the various P450 monooxygenase activities. These activities (data provided by Xenotech, LLC) included 7-ethoxyresorufin O-deethylation (CYP1A2), coumarin 7-hydroxylase (CYP2A6), Taxol 6-hydroxylase (CYP2C8), tolbutamide methyl-hydroxylase (CYP2C9), (S)-mephetynid 4'-hydroxylase (CYP2C19), chlorozoxazone 6-hydroxylase (CYP2E1), bufuralol 1'-hydroxylase (CYP2D6), and testosterone 6β-hydroxylase (CYP3A4/5). Correlation coefficients \((r)\) were determined graphically (Maclinist et al., 1995; Rodrigues et al., 1996). In each case, the statistical significance of the correlation is denoted by: * \(p < 0.05\), ** \(p < 0.01\), and *** \(p < 0.001\) (Student's \(t\) test).

**P450 Selective Inhibitors.** Inhibition studies with P450 form selective chemical inhibitors were carried out at a final [\(^{14}\)C]etoricoxib concentration of 5 \(\mu\)M (\(\text{IC}_{50}\)) and 200 \(\mu\)M (\(\text{IC}_{90}\)). Where possible, mechanism-based inhibitors (e.g., troleanidamycin and furafylline), relatively high affinity reversible inhibitors (\(K_i \approx 1.0 \mu\)M), or cosubstrates (\(K_m = 5.5 \mu\)M) were used. Where appropriate, the final concentration of the inhibitor (cosubstrate) exceeded (\(> 10\)-fold) its apparent \(K_i\) (Newton et al., 1995; Bourrie et al., 1996; Rodrigues...
et al., 1996). This ensured maximal inhibition (>80%) of each P450 form ([I]/[K]_{i} ≥ 10; [S] ≤ [K]_{i}). In addition, the inhibitors were dissolved in acetonitrile:water (50:50, v/v), in which case the final concentration of acetonitrile in the incubations was kept to a minimum (≤1.0% v/v).

**Incubation with Insect Cell Microsomes Containing cDNA-Expressed P450 Forms.** Incubations with [14C]etoricoxib (50 μM) were conducted at 37°C, in 1.5 ml polypropylene centrifuge tubes (final volume of 0.25 ml to 0.6 ml), and were conducted as described for liver microsomes, except that the molarity of phosphate buffer was decreased to 10 mM. Samples were preincubated for 5 min, after which time the reaction was started with the addition of rapidly thawed (37°C) microsomal protein (0.5 mg/ml final concentration). In all cases, the reactions were allowed to proceed for the maximum period of linearity (CYTP3A4 + h, 20 min; CYP1A2, 80 min; CYP2A6, 40 min; CYP2C9*1, 10 min; CYP2C8, 160 min; CYP2C19, 10 min; and CYP2D6, 10 min).

For all P450 proteins tested, the reaction rates (pmol/h/pmol of P450) were normalized (pmol/h/pmol · pmol of P450/mg) with respect to the corresponding nominal (mean) specific content of each P450 in native human liver microsomes (data provided by GENTEST Corp.; CYTP3A4, 108 pmol/mg; CYP1A2, 45 pmol/mg; CYP2A6, 68 pmol/mg; CYP2C9, 96 pmol/mg; CYP2C8, 64 pmol/mg; CYP2C19, 19 pmol/mg; and CYP2D6, 10 pmol/mg). The normalized rates (pmol/h/mg) were then added, and the normalized rate was calculated as the percentage of the total normalized rate (Rodrigues, 1999).

Apparent K_{i} and V_{max} were determined using an in-house preparation of insect cell microsomes containing cDNA-expressed CYTP3A4 (Mei et al., 1999). Incubations were performed in 100 mM potassium phosphate buffer (pH 7.4), as described for native liver microsomes (final concentration of CYTP3A4 = 0.3 nmol/ml).

**Immuno inhibition Studies.** [14C]Etoricoxib (50 μM) was incubated with a native human liver microsomes (pool of samples HHM-028H, HHM-028M, and HHM-0253) as described previously (0.24 mg protein/ml; 146 pmol of P450/ml). Incubations (final assay volume of 0.25 ml) were performed in the presence of increasing amounts (0–18 mg IgG/nmol of P450) of preimmune sera or anti-CYTP3A4 peptide polyclonal antibodies (Wang and Lu, 1997).

**HPLC.** Etoricoxib and its metabolites were separated on a reverse-phase C_{8} (Zorbax Eclipse XDB-C_{8}, 4.6 × 250 mm, 5 μm) column using a Hewlett-Packard HP1100 liquid chromatography system with the column oven temperature set at 40°C. The mobile phase consisted of (A) 20% acetonitrile:80% ammonium acetate (25 mM, pH 7–7.4), and (B) acetonitrile and was programmed to go from 0 to 30% B over 30 min at a flow rate of 1 ml/min. The effluent was monitored by photodiode array detection at 236 and 280 nm, and by an on-line radiometric detector (β-RAM, INUS Systems, Inc., Tampa, FL) using a 3 ml/min flow rate for the scintillation cocktail. Under these conditions, the retention times (≥0.5 min) of etoricoxib, M2 (6'-methoxy hydroxyl metabolite), M1 (1'-N-oxide metabolite), and M3 (6-carboxy metabolite) were 26, 17, 14, and 9 min, respectively.

**LC/MS.** Metabolites of etoricoxib were identified by electrospray LC/MS/MS analysis using the Finnigan LCQ mass spectrometer. The spray voltage was held at 4.1 kV, and the capillary temperature and voltage were set at 200°C and 6.0 V, respectively. Samples (microsomal and urine extracts) were dissolved in 30% aqueous acetonitrile and introduced into the mass spectrometer via a Zorbax eclipse XDB-C_{8} column. A gradient of 25 mM ammonium acetate and acetonitrile was used as the mobile phase. In positive ion mode MS, the 6'-hydroxymethyl metabolite (M2), yielded an MH^{+} ion (m/z 375), which upon MS/MS analysis produced intense fragment ions at m/z 357 (MH÷H_{2}O) and 278. Similarly, the N-oxide metabolite (M1) yielded an MH^{+} ion at m/z 375 and produced ions at m/z 357, 296, and 278. The MM/MS spectrum (precursor m/z 389; MH^{+}) of the carbonyl metabolite (M3) exhibited intense ions at m/z 343, 328, and 280. Both the production ion spectra and HPLC retention times of the microsomal and urinary metabolites were similar to those obtained from samples of authentic standards and confirmed earlier findings (Chauret et al., 2001).

**Effect of Etoricoxib on P450-Catalyzed Monooxygenase Activities in Native Human Liver Microsomes.** Etoricoxib (0.1, 1.0, 10, and 100 μM) was incubated with human liver microsomes, and its effect on various P450 form selective monooxygenase activities was determined. Activities (phenacetin O-deethylase, CYP1A2; diclofenac 4'-hydroxylase, CYP2C9; (S)-(+) -mephénytoin 4'-hydroxylase, CYP2C19; bufuralol 1'-hydroxylase, CYP2D6; chlorozoxone 6-hydroxylase, CYP2E1; and testosterone 6β-hydroxylase, CYP3A4/5) were measured using standard incubation protocols (Newton et al., 1995; Bourrie et al., 1996; Rodrigues et al., 1996). The following substrate concentrations were used: phenacetin, 25 μM; diclofenac, 10 μM; mephénytoin, 40 μM; bufuralol, 5 μM; chlorozoxone, 20 μM; and testosterone, 75 μM. For the purposes of comparison, positive control inhibitors were run in parallel (fluvoxamine, CYP1A2; sulfaphenazole, CYP2C9; omeprazole sulfate, CYP2C19; quinidine, CYP2D6; 4-methylpyrazole, CYP2E1; and ketoconazole, CYP3A4/5).

**Analysis of Human Urine.** Six healthy male subjects received an i.v. (25 mg, 100 μCi) dose of [14C]etoricoxib. The dose was administered in citrate-buffered saline (pH 3.6, 0.75 mg/ml) as a 15-min infusion. Urine (0–2, 2–4, 4–6, 6–9, 9–12, 12–18, and 18–24 h postdose) was collected and stored frozen at −20°C. Prior to analysis, the urine was thawed at room temperature, and aliquots from each time interval were combined in proportion to their respective volumes to produce a representative 0–24 h sample for each subject. Aliquots (0.2 ml) were taken for liquid scintillation counting. A second aliquot (5 ml) was transferred to glass centrifuge tubes, and trifluoroacetic acid (15 μl) was added to acidify the samples to approximately pH 3. Acetonitrile (20 ml) was added with mixing, and particulate material was removed by centrifugation (200 g, 10 min). The supernatants were transferred to 50-ml glass tubes, and solvent was removed in a centrifugal vacuum concentrator (Speed-Vac, Savant, Holbrook, NY). The residues were reconstituted in mobile phase and analyzed by HPLC with radiometric detection as described previously.

**Results**

**P450-Dependent Metabolism of Etoricoxib.** A typical radiochromatograph of the supernatant following incubation of [14C]etoricoxib with NADPH-fortified human liver microsomes is presented in Fig. 2A. After incubation, one major metabolite peak was observed (retention time of 17 min), which coeluted with authentic M2 standard and was identified by LC/MS as the 6'-methyl hydroxyl metabolite of etoricoxib. Omission of the NADPH generating system completely abolished the hydroxylation reaction by human liver microsomes, indicating that metabolite formation was enzymatic and NADPH-dependent. By comparison, low levels of the 1'-N-oxide metabolite (M1) were detected in the presence of the NADPH generating system (Fig. 2A). In addition, no secondary metabolism was detected, although the 6'-carboxy metabolite (M3) accounted for the majority of the radioactivity in human urine (Fig. 2B) and feces (R. Halpin unpublished observations).

In a panel of human liver microsomes (n = 15 different organ donors), the interindividual variability in the rate of 6'-methyl hydroxylation ranged from 3.5-fold at a final etoricoxib concentration of 200 μM (range 105–372 pmol/min/mg; mean of 203 pmol/min/mg) to 9.5-fold at a final etoricoxib concentration of 5 μM (range 3.2–18.5 pmol/min/mg; mean of 9.5 pmol/min/mg).

**Reaction Kinetics and Prediction of Intrinsic Clearance.** Under linear reaction conditions (1–1.5 mg protein/ml; 30-min incubation), the 6'-methyl hydroxylation of etoricoxib (1.0–1300 μM) in native liver microsomes corresponded to single K_{m} Michaelis-Menten kinetics (Fig. 3A). Nonlinear transformation of the data yielded a mean apparent K_{m} and V_{max} of 186 μM and 0.76 nmol/min/mg (mean of n = 3 different microsomal preparations), respectively (Table 1).

The in vitro intrinsic clearance (V_{max}/K_{m} ratio) was scaled, with respect to yield of microsomal protein and liver weight, and yielded a value of 3.1 to 9.7 ml/min/kg of b.wt. as the formation clearance of M2. Similarly, the in vivo intrinsic clearance was estimated to be 8.3 ml/min/kg, using i.v. data obtained from six subjects.

**Correlation Studies.** Etoricoxib (5 and 200 μM) hydroxylation was significantly correlated (r = 0.80–0.89; p < 0.001; n = 15) with CYTP3A4/5-selective testosterone 6β-hydroxylase activity in the bank of liver microsomes. By comparison (Table 2), the correlation with
7-ethoxyresorufin O-deethylase (CYP1A2), (S)-(−)-1-mephenytoin 4'-hydroxylase (CYP2C19), tolbutamide methyl hydroxylase (CYP2C9), chlorzoxazone 6-hydroxylase (CYP2E1), and bufuralol 1'-hydroxylase (CYP2D6) activity was relatively weak ($r^2 = 0.47$). It is important to note that, while the correlations with CYP2A6-selective coumarin hydroxylase ($r^2 = 0.71$; $p < 0.01$) and CYP2C8-selective Taxol 6-hydroxylase ($r^2 = 0.72$; $p < 0.01$) activities are significant, both activities correlated with the rates of testosterone 6β-hydroxylation ($r^2 > 0.79$; $p < 0.001$) and, because of coregression, correlation with etoricoxib hydroxylation would be expected. Moreover, correlation of etoricoxib hydroxylation activity with these enzymes did not improve in the presence of CYP3A4 monoclonal antibody (data not shown).

Overall, the data indicate that CYP3A subfamily member(s) play a major role in the formation of M2 over a relatively wide etoricoxib concentration range (5–200 μM). In agreement, the correlation of M2 formation at two different concentrations of etoricoxib (5 μM versus...
200 μM) was significant (r = 0.81; p < 0.001), also suggesting that the same P450 form(s) catalyzed the reaction over the etoricoxib concentration range studied.

**Chemical Inhibition Studies.** Ketoconazole (2.0 μM) and troleandomycin, both selective inhibitors of CYP3A in native human liver microsomes, were shown to effectively decrease (65–70%) the rate of M2 formation at two concentrations (5 and 200 μM) of etoricoxib (Fig. 4). By comparison, chemical inhibitors selective for other P450s were relatively weak, although some inhibition (~10%) was observed in the presence of furafylline (CYP1A2-selective), sulfaphenazole (CYP2C9-selective), or quinidine (CYP2D6-selective). In agreement with correlation studies, these data suggested that the 6′-methyl hydroxylation of etoricoxib was primarily catalyzed by member(s) of the CYP3A subfamily, although other P450s (~10% each), such as CYP1A2, CYP2D6, and CYP2C9 also played a role. When sulfaphenazole (5 μM), ketoconazole (2 μM), and quinidine (10 μM) were coincubated, M2 formation was inhibited by approximately 84 ± 5.2% (data not shown), suggesting that CYP3A, CYP2C9, and CYP2D6 accounted for the majority of the oxidative (NADPH-dependent) metabolism of etoricoxib in native human liver microsomes.

In a separate series of experiments, the inhibitory effect of troleandomycin was confirmed with the bank of human liver microsomes (Fig. 5). Inhibition in the presence of troleandomycin (mean ± S.D. = 63 ± 13.4; n = 16 subjects), which varied from 40% (subject 24) to 90% (subject 2), correlated (r = 0.87; p < 0.001) with the levels of CYP3A (testosterone 6β-hydroxylase) activity (data not shown). Despite a lower contribution from CYP3A, other P450s (e.g., CYP2C9, CYP2D6, or CYP1A2) did not contribute to more than 17% of the etoricoxib 6′-methyl hydroxylase activity in microsomes of subject 24 (data not shown).

**Immunoinhibition Studies with Native Human Liver Microsomes.** To confirm the P450 reaction phenotyping results obtained with chemical inhibitors and cDNA-expressed P450s, [14C]etoricoxib (50 μM) was incubated with native human liver microsomes in the absence and presence of immunoinhibitory anti-CYP3A4 peptide polyclonal antibodies. The anti-CYP3A4 antibody preparation has been shown to be highly selective for CYP3A4-catalyzed reactions in native human liver microsomes (Wang and Lu, 1997), and the results with etoricoxib indicated that the majority (~60%) of the 6′-methyl hydroxylase activity was attributable to CYP3A4 (Fig. 6).

**Metabolism by cDNA-Expressed P450 Forms.** Of the P450 forms tested, CYP3A4 (20 pmol/h/pmol of P450), CYP2D6 (95 pmol/h/pmol of P450), and CYP2C19 (16 pmol/h/pmol of P450) exhibited high rates of hydroxylase activity (Table 3). By comparison, the rate of hydroxylation in the presence of cDNA-expressed CYP1A2, CYP2A6, CYP2C8, CYP2C9, and CYP2E1 was low (≤2.3 pmol/h/pmol of P450). No activity was detected in (control) microsomes prepared from insect cells containing the selectable plasmid vector without cDNA insert (data not shown).

To obtain meaningful information, the turnover rates obtained with

**TABLE 2**

<table>
<thead>
<tr>
<th>Activity*</th>
<th>P450s</th>
<th>Correlation Coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 μM</td>
<td>200 μM</td>
</tr>
<tr>
<td>TESTase</td>
<td>CYP3A4/5</td>
<td>0.80***</td>
</tr>
<tr>
<td>ERODase</td>
<td>CYP1A2</td>
<td>0.31</td>
</tr>
<tr>
<td>COHase</td>
<td>CYP2A6</td>
<td>0.50</td>
</tr>
<tr>
<td>TAXase</td>
<td>CYP2C8</td>
<td>0.49</td>
</tr>
<tr>
<td>TOLase</td>
<td>CYP2C9</td>
<td>0.04</td>
</tr>
<tr>
<td>MEPHase</td>
<td>CYP2C19</td>
<td>0.15</td>
</tr>
<tr>
<td>BUFase</td>
<td>CYP2D6</td>
<td>0.34</td>
</tr>
<tr>
<td>CHLORase</td>
<td>CYP2E1</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* The various activities are TOLase, tolbutamide hydroxylase; COHase, COU hydroxylase; ERODase, 7-ethoxycoumarin O-deethylase; CHLORase, chlorzoxazone 6-hydroxylase; BUFase, bufuralol 1′-hydroxylase; TESTase, testosterone 6β-hydroxylase; MEPHase, (S)-(+)-mephénytoïn 4′-hydroxylase; and TAXase, Taxol 6-hydroxylase. The correlation coefficient was determined with the liver tissue of 15 different organ donor subjects. Statistical significance (Student’s t test) of the correlation is denoted by: **p < 0.01 and ***p < 0.001.
the various cDNA-expressed P450 proteins were normalized with respect to the nominal abundance of each P450 protein in native human liver microsomes (Shimada et al., 1994; Lasker et al., 1998; Rodrigues, 1999). It is evident from the data presented in Table 3 that the hydroxylation of etoricoxib is largely mediated by CYP3A4 (60%), whereas a lesser contribution is made by other P450s, such as CYP2C9, CYP2D6, and CYP2C19. In agreement, etoricoxib hydroxylation catalyzed by insect cell microsomes containing recombinant CYP3A4 was characterized by an apparent \( K_m \) (201 mM) similar to that obtained with native liver microsomes (Fig. 3). If the \( V_{\text{max}} \) (1.9 nmol/min/mg of CYP3A4) is normalized with respect to the mean specific content of CYP3A4 (~0.1 nmol/mg) in a bank of native human liver microsomes (Shimada et al., 1994), this yields a predicted \( V_{\text{max}} \) of 0.19 nmol/min/mg in human liver microsomes. As shown in Table 1, this value is very close to the \( V_{\text{max}} \) obtained with the pool of native human liver microsomes (sample HHM-0253, \( V_{\text{max}} \) 0.27 nmol/min/mg; 0.16 nmol/min/mg, when adjusted for the contribution of CYP3A, i.e., \( V_{\text{max}} \cdot f_{\text{in}}, \text{CYP3A4} = 0.27 \cdot 0.6 \)).

**Effect of Etoricoxib on P450 Form Selective Monooxygenase Activities in Native Human Liver Microsomes.** The inhibitory potential of etoricoxib (0.1–100 \( \mu M \)) toward six human liver P450 activities was evaluated. In all cases, etoricoxib was found to be a relatively weak inhibitor (IC\(_{50} \), \( > 100 \mu M \)). Data obtained with positive control inhibitors showed that incubation conditions were suitable for generating inhibition. IC\(_{50} \) values obtained with positive controls were as follows: quinidine (bufuralol 1'-hydroxylase), IC\(_{50} \) = 0.8 \( \mu M \); fluvoxamine (phenacetin O-deethylase), IC\(_{50} \) = 0.7 \( \mu M \); sulphasalazine (diclofenac 4'-hydroxylase), IC\(_{50} \) = 0.6 \( \mu M \); omeprazole sulfone (meprhenytoin 4'-hydroxylase), IC\(_{50} \) = 22 \( \mu M \); 4-methyl-

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**TABLE 3**

Metabolism of etoricoxib in the presence of insect cell microsomes containing various cDNA-expressed P450 proteins

<table>
<thead>
<tr>
<th>CYP</th>
<th>pmol/h/pmol pmol/h/mg</th>
<th>% of Total Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4 + ( b_1 )</td>
<td>20</td>
<td>2146</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>1.2</td>
<td>52</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>0.3</td>
<td>23</td>
</tr>
<tr>
<td>CYP2E1 + ( b_1 )</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>CYP2C9*1</td>
<td>2.3</td>
<td>222</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>0.2</td>
<td>15</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>95.1</td>
<td>951</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>16</td>
<td>304</td>
</tr>
<tr>
<td><strong>Total</strong>(^a)</td>
<td><strong>3712</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

N.D., no activity detected.

\(^a\) Hydroxylation of etoricoxib (10 \( \mu M \)) in the presence of each P450 form (pmol/h/pmol of CYP) was normalized with respect to the nominal level of each P450 form present in native human liver microsomes (data supplied by GENTEST Corp.; CYP1A2, 45 pmol/mg; CYP2C19, 19 pmol/mg; CYP3A5 assumed absent); 108 pmol/mg; CYP2D6, 10 pmol/mg; CYP2E1, 49 pmol/mg; CYP2A6, 68 pmol/mg; CYP2C9, 96 pmol/mg; and CYP2C8, 64 pmol/mg. Normalized rates are expressed as pmol/mg of microsomal protein. All insect cell microsome preparations contained overexpressed NADPH P450 reductase. However, CYP3A4 and CYP2E1 were also co-expressed with cytochrome \( b_6 \).

\(^b\) Sum of normalized rates.
CYP1A2, and possibly CYP2C19) more or less equally contribute to the major metabolic pathway in NADPH-fortified human liver microsomes (Fig. 2A). By comparison, 1’-N-oxidation to M1 is a relatively minor pathway that agrees with previously reported data (Chauret et al., 2001). The C-oxidation of etoricoxib (to the 6’-carboxy metabolite M3) is the predominant pathway in vivo (Fig. 2B) and suggests that generation of M3 (retention time ~10 min) in vitro requires the presence of cytoyscopic enzyme(s). In fact, M3 was shown to be formed by human liver S9 fraction in the presence of NAD+ (data not shown).

Because etoricoxib is extensively metabolized in humans (>90% of the dose), an attempt was made to predict the \( \text{CL}_{\text{int, in vivo}} \) based on estimates of apparent \( K_m \) and \( V_{\text{max}} \) in vitro (Table 1). The \( \text{CL}_{\text{int, in vitro}} \) obtained with human liver microsomes was scaled (Obach et al., 1997) to yield a predicted \( \text{CL}_{\text{int, in vivo}} \) of 3.1 (HHM-0253) to 9.7 (HHM-0228) mI/min/kg. These values compare favorably with the observed \( \text{CL}_{\text{int, in vivo}} \) (8.3 mI/min/kg; mean of \( n = 6 \) subjects; coefficient of variation = 25%; N. Agrawal unpublished results) calculated from the i.v. data (eq. 2).

In human liver microsomes, the 6’-methyl hydroxylation of etoricoxib was characterized as a relatively high apparent \( K_m \) process (~0.2 mM), a result that agrees well with earlier data (Chauret et al., 2001) and that has two clinical implications. First, etoricoxib would be expected to exhibit linear pharmacokinetics, which is in agreement with recent findings (N. Agrawal, unpublished observations). Second, one would expect etoricoxib (plasma \( C_{\text{max}} \) in humans ≤3 µg/ml; ≤8 µM) to be a weak competitive inhibitor of hepatic CYP3A4.

Several lines of evidence (e.g., correlation analysis, P450 form selective inhibitors, and cDNA-expressed P450 proteins) have demonstrated that member(s) of the CYP3A subfamily, most likely CYP3A4, is (are) the principal human liver microsomal enzyme(s) involved in the hydroxylation of etoricoxib. In fact, data obtained with a bank of liver microsomes (\( n = 16 \) different organ donors) suggested that the contribution of CYP3A varied from 40% to 90% (mean ± S.D. = 68 ± 13.4%; Fig. 5), which was in agreement with the results obtained with a pool of human liver microsomes (Fig. 4). However, it was not possible to evaluate the role of CYP3A5 (versus CYP3A4), because no attempt was made to measure the level of this enzyme in the microsomes used in this study, although the antipeptide antibody used for immunoinhibition studies has been shown to be selective (versus CYP3A5) for CYP3A4 (Wang and Lu, 1997).

Although the data point to a major role for CYP3A, results obtained with P450 form selective chemical inhibitors and recombinant proteins indicate that a number of other P450s (CYP2D6, CYP2C9, CYP1A2, and possibly CYP2C19) more or less equally contribute to the remainder (~40%) of the 6’-methyl hydroxylation activity in native human liver microsomes. Even when the contribution of CYP3A is relatively low, as in subject 24 (Fig. 5), CYP2D6, CYP2C9, or CYP1A2 do not contribute to more than 17% of the metabolism of etoricoxib (data not shown).

Thus, the P450 reaction phenotype of etoricoxib is rather unique and differs from those of other COX inhibitors, such as celecoxib, meloxicam, lornoxicam, ibuprofen, flurbiprofen, and indomethacin. All of these drugs are extensively metabolized in vivo and are primarily (~80%) metabolized (\( K_m \approx 50 \) µM) by a single P450 form (CYP2C9) in human liver microsomes (Bonnahby et al., 1996; Tracy et al., 1996; Hamman et al., 1997; Chesne et al., 1998; Nakajima et al., 1998; Tang et al., 2000).

For etoricoxib, the clinical relevance of the in vitro P450 reaction phenotype described herein will ultimately depend upon the fraction of the dose that is metabolized via 6’-methyl hydroxylation, which has been estimated to be ~80% (based on urinary and fecal profiles of subjects dosed with [14C]etoricoxib; R. Halpin, unpublished observations). The results of this study indicate that CYP3A4 accounts for 40 to 90% of the total 6’-methyl hydroxylation activity in human liver microsomes, which implies that approximately 30 to 70% of the dose would be cleared by the enzyme (\( K_m, \text{CYP3A} = 0.3–0.7 \) in vivo). Therefore, it is possible that the area under the curve of etoricoxib would be relatively refractory to the effects of CYP3A4 inhibitors (~3-fold increase) and inducers (~60% decrease) (Rodrigues and Wong, 1997).

Interestingly, despite the fact that multiple P450 forms were shown to catalyze the 6’-methyl hydroxylation of etoricoxib (1–1300 µM), the data obtained with native human liver microsomes were adequately described by a single \( K_m \) model (Fig. 3A). This finding indicates that CYP2C9, CYP2C19, CYP1A2, CYP2D6, and CYP3A4 are likely to be characterized by similar apparent \( K_m \) values. As expected, CYP3A4 predominates by virtue of its high abundance in the liver (Shimada et al., 1994). Although CYP2C9, CYP2C19, and CYP2D6 are known to be polymorphically expressed (Bertilsson, 1995; Guengerich, 1995; Nasu et al., 1997; Ruas and Lechner, 1997), each enzyme plays a relatively minor role (~10%) in the metabolism of etoricoxib. Therefore, the pharmacokinetic profile of etoricoxib is not expected to cosegregate with these polymorphisms.

In conclusion, etoricoxib is metabolized via 6’-methyl hydroxylation in human liver microsomes. The reaction is catalyzed by a number of P450 forms, although CYP3A4 accounts for the majority (40–90%) of the activity. The remainder of the activity is equally divided between a number of other P450s (e.g., CYP2D6, CYP2C9, CYP1A2, and possibly CYP2C19). In this regard, the P450 reaction phenotype of etoricoxib is unique and differs from that of other COX inhibitors.

Acknowledgments. We thank Dr. Dennis Dean (Radiosynthesis Group, Drug Metabolism Department, Merck Research Laboratories, Rahway, NJ) for synthesis of [14C]etoricoxib and Dr. T. Prueksaritanont (Drug Metabolism Department, Merck Research Laboratories, West Point, PA) for assistance with the PCNONLIN analysis. In addition, we are grateful to Dr. Regina Wang (Drug Metabolism Department, Merck Research Laboratories, Rahway, NJ) for providing the preparation of anti-CYP3A4 peptide polyclonal antibodies.

References


