ABSTRACT:

In vitro studies were conducted to identify the major metabolites of eplerenone (EP) and the cytochrome P450 (P450) isozymes involved in its primary oxidative metabolism in humans and dogs. The major in vitro metabolites were identified as 6β-hydroxy EP and 21-hydroxy EP in both humans and dogs. EP was metabolized by cDNA-expressed human CYP3A4 and dog CYP3A12 but only minimally by human CYP3A5. In human microsomes, inhibition of total metabolism by the CYP3A-selective inhibitors ketoconazole, troglodecymycin, and 6,7-dihydroxybergamottin, each at 10 μM concentration, was 83 to 95%, whereas inhibition with inhibitors selective for other P450 isozymes was minimal. In dog liver microsomes, the percentages of inhibition were 53 to 76% with the CYP3A-selective inhibitors. A monoclonal anti-CYP3A4 antibody inhibited EP metabolism by 84%, whereas other monoclonal antibodies had minimal effects. The formation of 6β-hydroxy and 21-hydroxy metabolites in human liver microsomes was best correlated with CYP3A-selective dextromethorphan N-demethylation and testosterone 6β-hydroxylation activities. EP moderately inhibited only CYP3A (testosterone 6β-hydroxylase) activity in human liver microsomes by 23, 34% and 45% at concentrations of 30, 100, and 300 μM, respectively. With human microsomes, the Vmax and Km for 6β-hydroxylation and 21-hydroxylation were 0.973 nmol/min/mg and 217 μM, and 0.143 nmol/min/mg and 211 μM, respectively. The human hepatic clearance calculated from total in vitro EP metabolism was 2.30 ml/min/kg, which agrees with in vivo data. In conclusion, 6β- and 21-hydroxylation of EP is primarily catalyzed by CYP3A4 in humans and CYP3A12 in dogs. Also, it is unlikely that EP would substantially inhibit the metabolism of other drugs that are metabolized by CYP3A4 or other P450 isoforms.
Materials and Methods

Materials. [14C]EP (lot numbers, GDS-8181-53A, GDS-8181-51A, GDS-8181-80B, and GDS-8181-28A, specific activity 16.5 or 125 μCi/mg, purity >95%), nonradiolabeled EP (lot numbers B900081 and 99K008-F3A) and 6β-hydroxyepiluprene (lot number GDS-6346-096A) were obtained from Pharmacia Corporation (Skokie, IL). Male and female dog liver microsomes were obtained from In Vitro Technologies (Baltimore, MD) and pooled equally for the appropriate experiments. Pooled human liver microsomes and the Reaction Phenotyping kit were obtained from XenoTech LLC (Kansas City, KS). Microsomes prepared from cDNA-transfected human B-lymphoblastoid cell lines that stably express CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, or CYP3A5, and several of their respective monoclonal or polyclonal antibodies, were obtained from BD Gentest Corporation (Woburn, MA). The specific CYP3A inhibitor, 6,7'-dihydroxybergamottin was synthesized by Pharmacia Corporation using the method reported by Bellevue et al. (1997). Dog CYP2D15 and CYP3A12 were cloned by Pharmacia Corporation using methods reported previously (Paulson et al., 1999). The recombinant dog P450 enzymes were expressed in S9 insect cells, commercially available from Invitrogen (Carlsbad, CA), using the bacmid methodology described by Luckow et al. (1993). [14C]-S-mephenytoin was obtained from Amersham Biosciences Inc. (Piscataway, NJ). All other chemicals and reagents were obtained from either Sigma-Aldrich (St. Louis, MO), ICN Biomedicals Inc. (Costa Mesa, CA), or Sigma-Urface Chemicals (Manchester, UK), and were of the highest quality available.

Metabolism of EP Using Human and Dog Microsomes. [14C]EP, prepared by combining appropriate amounts of radiolabeled and nonradiolabeled compound in an aqueous solution of acetone (1% v/v final concentration), was incubated with human and dog liver microsomes (1.5 mg/ml) and EP at concentrations of 3.62, 6.03, 36.2, 60.3, 121, 181, 241, 302 μM. Extracts were analyzed for production of 6β-hydroxy EP, 21-hydroxy EP, and disappearance of EP (total metabolism) using an HPLRC procedure. Mean metabolic rates were fit to an equation describing Michaelis-Menten kinetics using WinNonlin (Pharsight Corp., Mountain View, CA), and fmax and Km values were obtained. Intrinsic clearance (CLint) was calculated by the following equation, assuming 45 mg of microsomal protein/g liver (Houston and Carlile, 1997; Naritomi et al., 2001) and 26 g of liver per kg body weight (Davies and Morris, 1993).

The hepatic clearance (CLint) was calculated using the well stirred model as follows:

\[
\text{CL}_{\text{int}} = \frac{Q_{\text{H}} \cdot f_u \cdot \text{CL}_{\text{reu}}}{Q_{\text{H}} \cdot f_u + \text{CL}_{\text{reu}}}
\]

in which QH is hepatic blood flow in man (15 ml/min/kg; Rowland and Tozer, 1995) and fu is the fraction unbound in plasma (approximately 0.4 in the range of therapeutic concentration).

Metabolism of [14C]EP Using a Reaction Phenotyping Kit. The Reaction Phenotyping kit (XenoTech, LLC) contained 16 separate preparations of human liver microsomes from individual donors. Each microsomal preparation was characterized by the manufacturer for P450 enzyme activities using specific marker reactions including the O-dealkylation of 7-ethoxyresorufin (CYP1A2), the 7-hydroxylation of coumarin (CYP2A6), the deethylation of 7-ethoxy-4-trifluoro-methylcoumarin (CYP2B6), the 6β-hydroxylation of Taxol (CYP2C8), the methylhydroxylation of tolbutamide (CYP2C9), the 4'-hydroxylation of S-mephenytoin (CYP2C19), the O-demethylation of dextromethorphan (CYP3A4), the 6β-hydroxylation of testosterone (CYP3A4), and the 12-hydroxylation of lauric acid (CYP4A9/11). The samples were prepared for incubation as described earlier (final [14C]EP concentration of 10 μM, final protein concentration of 0.5 mg/ml, 15-min incubation). To facilitate the possible metabolism of EP by P450 isoforms other than CYP3A, the experiment was repeated in the presence of the CYP3A inhibitor ketoconazole in two different ways. The first was the same as outlined above, except ketoconazole was included at a final concentration of 10 μM. The second included ketoconazole at a final concentration of 10 μM, [14C]EP at a final concentration of 50 μM, and microsomal protein at a final concentration of 1.0 mg/ml in a 30-min incubation. In these cases, the samples were preincubated for 5 min with inhibitor before adding EP; to ensure maximal inhibition of the CYP3A isoforms. Concentrations of [14C]EP and metabolites were determined using an HPLRC procedure. Correlation coefficients (r), comparing the metabolic rates of EP in each microsomal preparation to the precharacterized P450 activities (data provided by XenoTech, LLC), were determined using Microsoft Excel 2000 (Microsoft Corporation, Seattle, WA).

Inhibition of EP Metabolism by Chemical Inhibitors. The effects of P450-selective chemical inhibitors on [14C]EP metabolism with liver microsomes were evaluated by determining the substrate metabolism rate in the absence (control) or presence of the following known isozyme-selective inhibitors: furafylline (CYP1A2), tranylcypromine (CYP2A6/2C19), orphenadrine (CYP2B6), sulfaphenazole (CYP2C9), quinidine (CYP2D6), dihydropycaïn...
(CYP2E1) and ketoconazole, 6,7-dihydroxybergamottin, and tololemorfin (CYP3A). The selective chemical inhibitors used to inhibit human P450 isoforms in human liver microsomes were also used to inhibit the same subfamily of dog P450 isoforms in dog liver microsomes. Chemical inhibitors were prepared in an aqueous solution of acetone. Control incubations contained the same final concentration of acetone (1%). Pooled human and dog liver microsomal samples were prepared and incubated (final [14C]EP concentration of 50 μM) as described previously. However, inhibitors were also included for a 10-min preincubation in the presence of the NADPH regenerating system prior to adding [14C]EP to allow for the maximal inhibition of P450 isoforms. The uniform inhibitor concentration (10 μM) was chosen to be at least 3 times higher than the Ki values found in the literature. Those inhibitors that were evaluated for selectivity at various concentrations were considered adequately so at 10 μM (Gannett et al., 1990; Newton et al., 1995; Bourrie et al., 1996; Bellevue et al., 1997; Sai et al., 2000; Parkinson, 2001; Taavitsainen et al., 2001). Following incubation, concentrations of [14C]EP and metabolites were determined using an HPLC procedure.

**Inhibition of EP Metabolism by Human P450 Antibodies.** Four P450-specific monoclonal antibodies (anti-CYP2A6, anti-CYP2D6, anti-CYP2E1, and anti-CYP3A4) and two polyclonal antibodies (anti-CYP1A2 and anti-CYP2C) were used to inhibit P450 isoforms in pooled human liver microsomes. Incubates were prepared and incubated (final [14C]EP concentration of 50 μM) as described previously, except that human liver microsomes and antibodies (1 mg of protein/ml) were preincubated for 15 min at 37°C before adding the substrate. Alternatively, control incubates contained an equivalent volume of 25 mM Tris buffer (pH of 7.4). Concentrations of [14C]EP and metabolites were determined using an HPLC procedure.

**Inhibition of Human P450 Isozyme Activities by EP.** Inhibition of P450 enzymes was measured with model substrates for cDNA-derived enzymes (BD Gentest Corporation) in microsomes prepared from human liver and subfamily of dog P450 isoforms in dog liver microsomes (CYP1A2, CYP2C9, CYP2D6, and CYP3A4) or from baculovirus-infected insect cells (CYP2C19). A single concentration (approximately 2 times apparent Km) of each model substrate was used, and their metabolic rates were determined by the measurement of an appropriate metabolite. Each enzyme assay included multiple EP concentrations separated by approximately 1/2 log (0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, and 300 μM) and two concentrations of a positive control inhibitor specific to the enzyme in question. Acetonitrile (final concentration 1%) was used as the substrate and inhibitor co-solvent. For this experiment, each enzyme assay required distinct incubation methods. These conditions were provided by BD Gentest Corporation and used with minor modifications. Briefly, the incubation conditions used for each enzyme assay were as follows:

All assays were performed in a final volume of 0.25 ml containing 3.3 mM MgCl2, 6 mM n-glucose-6-phosphate, 1.3 mM NADP+, and 0.4 U/ml n-glucose-6-phosphate dehydrogenase (Sigma-Aldrich, type VII from baker’s yeast), and incubated at 37°C in a shaking water bath for 30 min. CYP1A2 catalytic activity was measured by the deethylation of phenacetin (50 μM) in incubates containing 0.4 mg/ml CYP1A2 enzyme protein and 0.1 M potassium phosphate, pH 7.4. Reactions were stopped by adding 50 μl of acetonitrile. The positive control inhibitor used was 7,8-benzoflavone at final concentrations of 0.3 and 3 μM, in the appropriate samples.

CYP2C9 activity was measured by the 4′-hydroxylation of diclofenac (6 μM) in incubates containing 0.02 mg/ml CYP2C9 enzyme protein and 0.1 M Tris buffer, pH 7.5. Reactions were stopped by adding 50 μl 94% acetonitrile, 6% acetic acid. The control inhibitor sulfaphenazole was included at concentrations of 0.3 and 3 μM.

CYP2C19 activity was measured by the 4′-hydroxylation of [14C]-s-mephenytoin (50 μM, specific activity 5.16 μCi/μmol) in incubates containing 0.08 ng/ml CYP2C19 enzyme protein and 50 mM potassium phosphate buffer, pH 7.4. Reactions were stopped by adding 50 μl of acetonitrile. The control inhibitor tranylcypromine was included at final concentrations of 0.3 and 3 μM.

CYP2D6 activity was measured by the 1′-hydroxylation of (2′)-bufuralol (10 μM) in incubates containing 0.1 mg/ml CYP2D6 enzyme protein and 0.1 M potassium phosphate buffer, pH 7.4. Reactions were stopped by adding 25 μl 70% perchlorate. The control inhibitor quinidine was included at final concentrations of 0.1 and 1 μM.

CYP3A4 activity was measured by the 6β-hydroxylation of testosterone (120 μM) in incubates containing 0.2 mg/ml CYP3A4 enzyme protein and 0.1 M potassium phosphate buffer, pH 7.4. Reactions were stopped by adding 125 μl of acetonitrile. The positive control inhibitor ketoconazole was included at concentrations of 0.1 and 1 μM.

**HPLRC.** An aliquot (200 μl) of sample was injected into an HPLRC system comprised of a Hewlett Packard series 1050 autosampler and pump (Hewlett Packard Analytical Direct, Wilmington, DE), equipped with an Alttech Altima C18 guard column (5 μ, 7.5 × 4.6 mm) and an Alttech Altima C18 HPLC column (5 μ, 150 × 4.6 mm) (Alttech Associates, Deerfield, IL). A linear gradient system was used consisting of acetonitrile and water, from 10:90 by volume to 50:50 over 40 min, at a flow rate of 1.0 ml/min. The column was re-equilibrated with mobile phase A for 15 min between injections. A Radiomatic Flo-One/Netra series A-500 radiochemical detector (PerkinElmer Life Sciences, Boston, MA), pumping Packard Flo-Scint III at 3 ml/min, detected the radiochromatographic profiles. Assay sensitivity of the system allowed for the detection of any radioactive metabolic peak areas greater than 1% of the total sample radioactivity.

**LC-MS.** Identification of the hydroxy metabolites of EP was performed on a Finngan TSQ-700 triple quadrupole LC-MS system (Thermo Finnigan, San Jose, CA) equipped with an atmospheric pressure chemical ionization unit. The corona current was set to 5 μA, the vaporizer temperature to 500°C, the capillary temperature to 200°C, the sheath gas to 80 psi, and the auxiliary gas to 10 ml/min. Samples were scanned at a mass range of 70 to 750 amu every 2 s. Samples were injected onto a 3.5 μ, 3 × 150 mm Zorbax SB-C18 LC column (MAC-MOD Analytical Inc., Chadds Ford, PA) at 50°C. The mobile phase gradient consisted of a 40-min ramp from 100% mobile phase A (95% H2O, 5% MeOH, 12.5 mM ammonium acetate, pH 4.5) to 100% mobile phase B (5% H2O, 95% MeOH, 12.5 mM ammonium acetate, pH 4.5).

**Determination of P450 Marker Substrates.** Methodologies for determination of P450 marker substrate metabolites were provided by BD Gentest Corporation and used with minor modifications. Briefly, samples aliquots were injected into an HPLC system using a Waters model 510 pump and a Waters model 717 plus autosampler (Waters, Milford, MA). Separation occurred in 45°C and 1 ml/min on a Sulpelo 4.6 × 250 mm Nucleosil C18, 5 μ HPLC column (Bellefonte, PA), with linear mobile phase gradients. Differing mobile phases, gradients of water and methanol, or water, acetonitrile, and perchlorate were used, depending on the analyte. A Waters model 486 tunable absorbance detector monitored for the metabolites acetaminophen (absorbance at 244 nm), 4′-hydroxydichlofenac (absorbance at 280 nm), and 6β-hydroxytestosterone (absorbance at 254 nm). A Packard model 150TR Radiomatic Flow-One flow scintillation analyzer (PerkinElmer Life Sciences) was used to detect [14C]-hydroxytestosterone. A Focis System 3 scanning spectrophotometer (Optical Technology Devices, Inc., Elmsford, NY) monitored for 1′-hydroxybufuralol (excitation at 252 nm and emission at 302 nm). When using absorbance or fluorescence methods, analytes were quantified by comparison to an appropriate standard curve.

**Results**

**Metabolism of EP In Liver Microsomes and cDNA-Expressed P450 Isozymes.** Metabolic profiles of [14C]EP after incubation with human and dog liver microsomes are shown in Fig. 2, A and B. The metabolite peaks eluting at the HPLC retention time of 22 to 22.5 min and 23 to 23.5 min were identified as 6β-hydroxy EP and 21-hydroxy EP, respectively, as described below. The major metabolite of [14C]EP produced with human microsomes was 6β-hydroxy EP, and a relatively small amount of 21-hydroxy EP was also found. With dog microsomes, the major metabolites were also 6β-hydroxy and 21-hydroxy EP.

Following a 1-h incubation in various cDNA-expressed isozyms, extensive metabolism of [14C]EP was observed with CYP3A4 (approximately 35% in Fig. 2C). Minimal metabolism (<1%) was observed in CYP3A5 (data not shown) whereas 50 μM [14C]testosterone, a positive control, showed extensive metabolism (>30%) with both CYP3A4 and CYP3A5 after a 10-min incubation. Finally, there was no apparent metabolism with CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP2E1 (metabolic
profiles not shown). Metabolic profiles from CYP3A4 incubates were similar to that in human liver microsomes. The major in vitro metabolites with human liver microsomes or CYP3A4 were 6β-hydroxy and 21-hydroxy EP, which accounted for greater than 90% of the total metabolism in incubates.

From the large-scale CYP3A4 incubation, the metabolite peaks eluting at the HPLC retention time of 22 to 22.5 min and 23 to 23.5 min were identified as 6β-hydroxy EP and 21-hydroxy EP by an LC-MS procedure comparing their HPLC retention times and MS fragmentation pattern with those of human in vivo metabolites previously identified by both NMR and LC-MS/MS. The atmospheric pressure chemical ionization LC-MS scans (Fig. 3) for the metabolites at retention times 22 to 22.5 min and 23 to 23.5 min were the same as those of the reference compounds 6β-hydroxy and 21-hydroxy EP, respectively. With dog P450 isozymes, the major metabolites were identified by HPLC retention time comparison. Substantial formation of 6β-hydroxy and 21-hydroxy EP was observed with CYP3A12 (Fig. 2D). However, there was no apparent metabolism with CYP2D15 (metabolic profile not shown). The metabolic profile from CYP3A12 incubates was similar to that in dog liver microsomes, although quantitative differences among the metabolite peaks were observed.

**Kinetics of EP Metabolism.** The $V_{\text{max}}$ and $K_{\text{m}}$ (± S.E.) measured from 6β-hydroxylation and 21-hydroxylation were 0.973 ± 0.132 nmol/min/mg and 217 ± 56.0 μM, and 0.143 ± 0.035 nmol/min/mg and 211 ± 101 μM, respectively (Fig. 4). The corresponding values, as measured from the disappearance of EP (total metabolism), were 1.76 ± 0.24 nmol/min/mg and 303 ± 70.0 μM. Even though enough curvature was present in the data to obtain estimated $V_{\text{max}}$ and $K_{\text{m}}$ values using WinNonlin, solubility of EP was the major limitation in determining metabolic rates at concentrations higher than 300 μM, thereby causing a relatively large standard error in their estimation. The CL$_{\text{int}}$ for 6β-hydroxylation, 21-hydroxylation, and total metabolism of EP were 5.24, 0.79, and 6.78 ml/min/kg, respectively, when calculated from the $V_{\text{max}}$ and $K_{\text{m}}$ values. Using CL$\text{int}$ and hepatic blood flow, the estimated CL$\text{H}$ of EP due to 6β-hydroxylation, 21-hydroxylation, and total metabolism were 1.84, 0.31, and 2.30 ml/min/kg. In this respect, CL$\text{H}$ measured from 6β-hydroxy and 21-hydroxy metabolites alone accounted for 93.4% of total EP CL$\text{H}$ as estimated using human liver microsomes.

**Correlation with P450 Isozyme Activities.** The metabolic rate of [14C]EP was correlated with specific P450 isozyme activities from human liver microsomes in the Reaction Phenotyping kit (Table 1). The total rate of [14C]EP metabolism was best correlated with the dextromethorphan N-demethylation (CYP3A) and testosterone 6β-hydroxylation (CYP3A) rates compared with rates of other marker metabolic reactions. When metabolism of EP to the two major metabolites was compared with P450 isozyme activities, the formation rates of both 6β-hydroxy and 21-hydroxy EP were best correlated with CYP3A activity. In this experiment, [14C]EP also seemed to correlate moderately (an $r$ value of approximately 0.5) with coumarin 7-hydroxylation (CYP2A6), 7-ethoxy-4-trifluoromethylcoumarin O-dealkylation (CYP2B6) and paclitaxel 6β-hydroxylation (CYP2C8). To verify whether these isoforms play a minor role in EP metabolism and whether the correlation improves by inhibition of CYP3A activities, the phenotyping experiment was repeated in the presence of ketoconazole. With ketoconazole, 10 μM EP metabolism was com-
pletely inhibited, and metabolites were not measurable in any of the microsomal preparations. Therefore, correlation values could not be determined. Using the modified conditions described earlier (i.e., using 50 μM EP, 1 mg protein/ml and 30-min incubation), moderate metabolism was observed in the presence of ketoconazole. In this case, the metabolic rates tended to be reduced by 90% when normalized for initial substrate concentration, and both 6β-hydroxy and 21-hydroxy metabolites were observed. However, the correlation values calculated for 6β-hydroxylation, 21-hydroxylation and total metabolism remained the same as in the experiment without ketoconazole. That is, correlations with CYP3A activity remained greater than 0.9, whereas correlations with CYP2A6, CYP2B6, and CYP2C8 activities remained approximately 0.5.

**Chemical Inhibition.** Inhibition of metabolism of [14C]EP in human liver microsomes was examined in the presence of various chemical inhibitors at a concentration of 10 μM. Total metabolism was markedly reduced with CYP3A inhibitors ketoconazole (95%), troleandomycin (83%), and 6',7'-dihydroxybergamottin (86%). When formation of 6β-hydroxy EP and 21-hydroxy EP was measured individually, metabolism of [14C]EP to these metabolites was significantly inhibited by ketoconazole, troleandomycin, and 6',7'-dihydroxybergamottin (Fig. 5, A and B). Formation of 6β-hydroxy EP was also significantly reduced by dihydrocapsaicin (31%) compared with control.

Inhibition of [14C]EP metabolism in dog liver microsomes was also examined in the presence of the various chemical inhibitors. With the dog microsomes, total metabolism of [14C]EP was markedly reduced only with CYP3A-selective inhibitors ketoconazole (66%), troleandomycin (76%), and 6',7'-dihydroxybergamottin (53%). When formation of 6β-hydroxy and 21-hydroxy EP was measured individually, metabolism of [14C]EP to these two metabolites was also significantly inhibited only by ketoconazole, troleandomycin, and 6',7'-dihydroxybergamottin (Fig. 5, C and D).

**Immunoinhibition of 6β- and 21-Hydroxy EP Formation.** Inhibition of [14C]EP metabolism in human microsomes was examined with human P450 antibodies. The total metabolism of [14C]EP was
inhibited by 84% with a monoclonal anti-CYP3A4 antibody. Inhibition of total metabolism of EP was minimal when human liver microsomes were incubated with other antibodies. When formation of 6β/H₉₂₅₂-hydroxy and 21-hydroxy EP was measured individually, metabolism of [¹⁴C]EP to these two metabolites was also significantly inhibited only by anti-CYP3A4 (Fig. 6).

**Inhibition of P450 Isozyme Activities by EP.** As indicated by metabolism of the marker substrates, EP at concentrations up to 300 µM did not remarkably reduce (<20% reduction) the activities of CYP1A2, CYP2C9, CYP2C19, or CYP2D6 (Fig. 7). Positive control inhibitors for these enzymes, however, did markedly reduce metabolism of marker substrates, indicating proper function of the assay. On the other hand, EP inhibited CYP3A4 catalytic activity by approximately 34% at 100 µM and approximately 45% at 300 µM. The IC₅₀

---

**TABLE 1**

<table>
<thead>
<tr>
<th>P450 Isozyme</th>
<th>Marker Reaction</th>
<th>Correlation (r)</th>
<th>Total Metabolism</th>
<th>6β-Hydroxylation</th>
<th>21-Hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>7-Ethoxyresorufin O-dealkylation</td>
<td>0.118</td>
<td>0.109</td>
<td>0.157</td>
<td></td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin 7-hydroxylation</td>
<td>0.463</td>
<td>0.494</td>
<td>0.519</td>
<td></td>
</tr>
<tr>
<td>CYP2B6</td>
<td>7-EFC  O-dealkylation</td>
<td>0.526</td>
<td>0.555</td>
<td>0.586</td>
<td></td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Paclitaxel (Taxol) 6β-hydroxylation</td>
<td>0.520</td>
<td>0.532</td>
<td>0.589</td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide methyl-hydroxylation</td>
<td>0.390</td>
<td>0.397</td>
<td>0.379</td>
<td></td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-mephenytoin 4′-hydroxylation</td>
<td>0.198</td>
<td>0.224</td>
<td>0.214</td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan O-demethylation</td>
<td>0.121</td>
<td>0.137</td>
<td>0.141</td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>0.363</td>
<td>0.308</td>
<td>0.305</td>
<td></td>
</tr>
<tr>
<td>CYP3A</td>
<td>Dextromethorphan N-demethylation</td>
<td>0.787</td>
<td>0.807</td>
<td>0.804</td>
<td></td>
</tr>
<tr>
<td>CYP3A</td>
<td>Testosterone 6β-hydroxylation</td>
<td>0.988</td>
<td>0.980</td>
<td>0.951</td>
<td></td>
</tr>
<tr>
<td>CYP4A9/11</td>
<td>Lauric acid 12-hydroxylation</td>
<td>0.044</td>
<td>0.065</td>
<td>0.111</td>
<td></td>
</tr>
</tbody>
</table>

* 7-Ethoxy-4-trifluoromethylcoumarin.

---

*Fig. 5. Inhibition of 6β-hydroxy EP (A) and 21-hydroxy EP (B) formation in human liver microsomes and 6β-hydroxy EP (C) and 21-hydroxy EP (D) formation in dog liver microsomes by P450 chemical inhibitors at 10 µM.*

Data are expressed as a percent of control values; CON, control; FUR, furafylline; TRA, tranylcypromine; ORP, orphenadrine; SUL, sulfaphenazole; QUL, quinidine; DIH, dihydrocapsaicin; KET, ketoconazole; DHB, 6/7,7-dihydroxybergamottin; TAO, troleandomycin. Error bars indicate standard error of the mean for an n of 3. * p < 0.05; **, p < 0.01; ***, p < 0.001.
for this interaction was not calculated because all inhibition values were below 50%. CYP3A activity was inhibited more than 75% by 0.1 μM and nearly 95% by 1.0 μM ketoconazole, the positive control inhibitor.

Discussion

The present study demonstrated that 6β- and 21-hydroxy EP are the major in vitro metabolites in both human and dog and that the metabolism to these hydroxy compounds was primarily mediated by CYP3A4 in humans and CYP3A12 in dogs. The involvement of CYP3A4 in the metabolism of EP in humans is well supported by the following findings: 1) metabolism of [14C]EP to 6β- and 21-hydroxy EP was best correlated with dextromethorphan N-demethylation and testosterone 6β-hydroxylation activities, which are typical markers for CYP3A activity; 2) correlation with other P450 marker reactions did not improve in the presence of CYP3A-selective inhibitor ketoconazole; 3) metabolism to both 6β- and 21-hydroxy EP was significantly inhibited by only CYP3A-selective inhibitors ketoconazole, troleandomycin, and 6',7'-dihydroxybergamottin; 4) metabolic activity was extensively inhibited by human anti-CYP3A4 antibody, but not by other antibodies; 5) EP was extensively metabolized to 6β- and 21-hydroxy [14C]EP by human cDNA-expressed CYP3A4 and only minimally by CYP3A5, but not by other CYP isoforms. With dog liver microsomes, metabolism of [14C]EP to both 6β- and 21-hydroxy EP, was inhibited by CYP3A specific inhibitors, ketoconazole, troleandomycin, and 6',7'-dihydroxybergamottin, whereas the metabolism was not inhibited by the inhibitors for other P450 isoforms. Furthermore, metabolism of [14C]EP was observed with dog cDNA-expressed CYP3A12, and there was no notable metabolism with dog CYP2D15. These results indicate that formation of 6β- and 21-hydroxy [14C]EP in the dog was also primarily mediated by CYP3A.

The chemical inhibitors used in this study were chosen based on a review of literature that ascertained their potency and selectivity. Nevertheless, in P450-selective chemical inhibition experiments with human liver microsomes, dihydrocapsaicin, a selective CYP2E1 inhibitor, reduced metabolism of [14C]EP significantly at a concentration of 10 μM (Fig. 5). However, there was no apparent metabolism with cDNA-expressed CYP2E1, EP metabolism (alone or in the presence of ketoconazole) was not well correlated with CYP2E1 activities (Table 1), nor was EP metabolism inhibited by anti-CYP2E1 (Fig. 3). Therefore, the minor reduction of EP metabolism by dihydrocapsaicin may be nonselective at the concentrations studied.

Examining EP metabolism using the Reaction Phenotyping kit of human microsomes revealed some moderate correlations with CYP2A6, CYP2B6, and CYP2C8 activities ($r$ values of approximately 0.5), in addition to higher correlations with CYP3A3 activities. These correlations were not improved in the presence of ketoconazole. Thus, these P450 isoforms were not involved in EP metabolism in vitro. Instead, these correlations appear to be artifacts of the microsomal samples chosen by Xenotech for this particular version of the Reaction Phenotyping kit. Using the marker reaction data alone, Xenotech determined that a selective CYP3A4/5 substrate reaction such as testosterone 6β-hydroxylation would have an apparent correlation of 0.46, 0.55 to 0.58, and 0.57 to marker reactions for CYP2A6, CYP2B6, and CYP2C8 activities, respectively (data provided by Xenotech). If EP were a substrate of one of these isoforms, a correlation value of significantly higher than 0.5 to 0.6 is expected, particularly in the presence of ketoconazole. This demonstrates the im-

![Fig. 6. Inhibition of EP metabolism to 6β-hydroxy EP and 21-hydroxy EP in human liver microsomes by P450 antibodies.](image)

Data are expressed as a percent of control values. Error bars indicate standard error of the mean for an $n$ of 3. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

![Fig. 7. Inhibition of metabolism of marker substrates for CYP1A2 (PHE, phenacetin), CYP2C9 (DIC, diclofenac), CYP2C19 (MEP, (S)-mephenytoin), CYP2D6 (BUF, bufuralol), and CYP3A4 (TES, testosterone) by EP at concentrations of 30, 100, and 300 μM (A) and by isozyme-specific positive control inhibitors (B) at the low and high concentrations.](image)

BEN, 7,8-benzoflavone for CYP1A2; SUL, sulfaphenazole for CYP2C9; TRA, tranylcypromine for CYP2C19; QUI, quinidine for CYP2D6; KET, ketoconazole for CYP3A4. The low and high concentrations are 0.3 and 3.0 μM for BEN, SUL and TRA, respectively, and 0.1 and 1.0 μM for QUI and KET, respectively. Values are means of $n = 2$. 


portance of evaluating the correlations between the marker metabolic reactions prior to final interpretation of the results from this type of phenotyping experiment.

A CLH4 value of 2.3 ml/min/kg was obtained from the total rate of EP metabolism using the well stirred model. The plasma clearance of EP after oral administration (CL/F) to humans was 2.7 ml/min/kg (C. S. Cook, L. M. Berry, A. Karim, J. Qian, M. Lonien, J. Hribar, R. Bible, unpublished data). Since F (fraction bioavailable) is expected to be greater than 0.7, and EP is extensively metabolized in humans, the in vivo hepatic clearance will be between 1.9 and 2.7 ml/min/kg. Thus, despite the solubility limitations in determining $\gamma_{max}$ and $K_{m}$, the CLH4 predicted by the in vitro values is in close agreement with in vivo data.

Steroid molecules such as testosterone and progesterone are metabolized to 6β-hydroxy metabolites by CYP3A3 (Yamazaki and Shimada, 1997), and it is not surprising to find that 6β-hydroxylation of EP is also catalyzed by CYP3A4. However, 6β-hydroxylation of testosterone and progesterone was also mediated by CYP2C9 and 2C19 although the rates of metabolism by these isozymes were much lower than those by CYP3A. Unlike these steroid molecules, there was no evidence that 6β-hydroxy EP was formed by these isozymes at the assay sensitivity level of 0.08 pmol/min/mg of protein. Moreover, EP was not substantially metabolized in the presence of cDNA-expressed CYP3A5, demonstrating that EP metabolism is highly selective to CYP3A4. Some steroid molecules, such as testosterone and estradiol, are good substrates of both CYP3A5 and CYP3A4, although the rates of metabolism of these compounds tended to be lower with CYP3A5 (Williams et al., 2002). In addition to 6β-hydroxylation, EP was also metabolized to 21-hydroxy EP, which has a hydroxyl group on the γ-lactone ring. To our knowledge, EP is the first example that demonstrates involvement of CYP3A4 in the hydroxylation of a γ-lactone ring. Hydroxylation of a γ-lactone ring has been shown for carrenone (Karim, 1978) and the naphthalenic lignan lactone 5-lipoxygenase inhibitor, 1,7,10,1253 (Chauvet et al., 1995). However, the P450 isoyme responsible for this type of metabolism has not been reported.

Even though the in vitro metabolites, 6β-hydroxy EP and 21-hydroxy EP, were formed by CYP3A3 subfamily enzymes in dogs, as observed in humans, the proportion of these two metabolites in dogs was substantially different from that in human. The major in vitro metabolite of EP formed in human liver microsomes was 6β-hydroxy EP, whereas only a small amount of 21-hydroxy was formed. In dog liver microsomes, on the other hand, there was relatively more 21-hydroxy EP formed than 6β-hydroxy EP. These results are in agreement with in vivo findings in which 6β-hydroxy EP is the major metabolite in humans (C. S. Cook, L. M. Berry, A. Karim, J. Qian, M. Lonien, J. Hribar, R. Bible, unpublished data), whereas in dogs, 21-hydroxy EP is the major metabolite (Cook et al., 2000). CYP3A4 and CYP3A12 share 79% amino acid sequence similarity. However, potentiating CYP3A4 inhibitors like ketoconazole, 6β,17α-dihydroxybergen, and troleandomycin significantly reduced EP metabolism. Therefore, it is anticipated that other potent CYP3A4 inhibitors (e.g., erythromycin, fluconazole, cyclosporin, saquinavir) will also reduce the metabolism of EP, thereby potentially increasing in vivo exposure to EP.

In summary, the results of the present study indicate that EP was primarily metabolized to 6β- and 21-hydroxy metabolites by CYP3A4 in humans and by CYP3A12 in dogs. Additionally, it is highly unlikely that EP will reduce the metabolism of other drugs, whereas only very potent CYP3A4 inhibitors are expected to substantially reduce the metabolism of EP in vivo.

Acknowledgments. The authors thank Dr. G. Mather at Cedra Corporation for conducting the kinetic studies of EP metabolism, Dr. W. F. Bushby, Mr. J. M. Ackermann, and Dr. D. M. Stresser at Gentest for conducting the inhibition study of CYP isozyme activities, and Mr. C. J. Gresk for coordinating the above two studies.

References


