We describe the kinetics of pentoxifylline formation from lisofylline in human liver microsomes using selective inhibitors of cytochrome P450 isozymes, correlation studies with specific isozyme activities, and cDNA-expressed human CYP1A2 and 2E1. A biphasic model fitted the data best for the formation of pentoxifylline, \( K_m = 0.282 \pm 0.135 \mu M \), \( V_{max1} = 0.003 \pm 0.001 \text{ nmol/min/mg protein} \), \( K_{mm} = 158 \pm 42.6 \mu M \) and \( V_{max2} = 0.928 \pm 0.308 \text{ nmol/min/mg} \) (N = 4). Pentoxifylline formation by the low \( K_m \) isozyme (200 \( \mu M \) lisofylline) required NADPH, was not inhibited by any isozyme-specific P450 inhibitor, and was inhibited only 10% and 20%, respectively, by aminobenzotriazole and N-octanoylamylamine. We concluded that the low \( K_m \) enzyme was not a cytochrome P450. At 5 \( \mu M \) of lisofylline the CYP1A2 inhibitor, furafylline, inhibited pentoxifylline formation by 58.8%, and the nonspecific CYP2E1 inhibitor, diethylthiocarbamate, inhibited pentoxifylline formation by 21.7%. When preincubated with furafylline plus diethylthiocarbamate, inhibition of pentoxifylline formation was increased 71.4%. Microsomal CYP1A2 activity correlated with pentoxifylline formation \( (r^2 = 0.870, p < 0.001) \). However, CYP2E1 activity did not correlate with pentoxifylline formation \( (r^2 = 0.143, p = 0.181) \). Baculovirus insect cell expressed human CYP1A2 formed pentoxifylline at 0.987 nmol/min/nmol cytochrome P450 at 5 \( \mu M \) lisofylline. cDNA expressed CYP2E1 did not catalyze formation of pentoxifylline. Diethylthiocarbamate inhibited pentoxifylline formation by 85.7% in cDNA expressed CYP1A2. We conclude that CYP1A2 is the high affinity enzyme catalyzing pentoxifylline formation from lisofylline.
Chemical Inhibition Studies. Studies to determine the effect of cytochrome P450 isoform-selective inhibitors on the human microsomal oxidation of lisofylline to pentoxifylline were undertaken with 5 μM lisofylline in five human livers. The inhibitors used and the isozymes inhibited were: furafylline (CYP1A2) (18–20), coumarin (CYP2A6) (21), orphenadrine (CYP2B6 and nonspecific) (22), tolbutamide (CYP2C8/9/10) (23, 24), sulfaphenazole (CYP2C9/10) (20, 25, 26), S-mephenytoin (CYP2C19, 500 μM) (27, 28), quinidine (CYP2D6) (29, 30), and diethylthiocarbamate (CYP2E1 and minor effects on CYP2A6 and CYP1A2) (20, 31–33), and troleandomycin (CYP3A) (33, 34). Concentrations used in the incubation were selected to inhibit 80% of the respective isoform activity while retaining specificity (20, 35). Poorly water soluble inhibitors (troleandomycin, tolbutamide, and sulfaphenazole) were initially dissolved in acetone, the appropriate amount of inhibitor was added to the incubation mixture which had been stopped by the addition of 6 ml methylene chloride. The tubes were shaken on a reciprocal shaker for 10 min and the有机 phase was transferred to a second tube and evaporated to dryness under N2. Mechanism-based inhibitors (furafylline, tolbutamide, diethylthiocarbamate, and orphenadrine) were preincubated with microsomes and NADPH for 15 min at 37°C. In these incubations, pentoxifylline formation was initiated by adding lisofylline and NADPH. To examine the general participation of P450, 1-aminobenzotriazole (1 mM) and dithiocarbamate, and orphenadrine were preincubated with microsomes and NADPH for 15 min at 37°C. In these incubations, pentoxifylline formation was initiated by adding lisofylline and NADPH. To examine the general participation of P450, 1-aminobenzotriazole (1 mM) and N-octamylamine (3 mM) were used. Aminobenzotriazole was dissolved in acetone, and N-octamylamine was diluted in methanol. Final methanol concentration in incubations was 2%. All incubations with inhibitors were performed in triplicate.

HPLC Analysis. Pentoxifylline formed in incubations was assayed by adding 100 μl (1 μg in H2O) of internal standard (CT-2410 R) to the incubation mixture which had been stopped by the addition of 6 ml ice-cold methylene chloride. The tubes were shaken on a reciprocal shaker for 10 min and centrifuged at 3,000 g for 10 min. The aqueous phase was aspirated to waste and the organic phase was transferred to a second tube and evaporated to dryness at 50°C under N2. The residue was reconstituted in 100 μl mobile phase and the 30–60 μl supernatant of pentoxifylline was pentoxifylline injected onto the HPLC.

The HPLC system consisted of a Beckman pump (114M solvent Delivery Module), Hewlett-Packard 1050 auto sampler (Hewlett-Packard Co., Wilmington, DE), Spectroflow 757 UV detector (ABI Analytical Kratos Division), and a C18 reversed-phase column (Microsorb-MV, 3 μm, 4.6 × 100 mm, Rainin Instrument Co. Inc., Woburn, MA) preceded by an Opti-Guard guard column (Optimize Technologies Inc., Portland, OR). The mobile phase was 25 mM ammonium phosphate buffer containing 0.25% acetic acid (pH 4.5 adjusted with ammonium hydroxide) : methanol, 65:35, delivered at a flow rate of 0.7 ml/min. The eluate was monitored at 273 nm. The retention times for pentoxifylline, lisofylline, and internal standard were 9.3, 13.5, and 20 min, respectively. The limit of detection for pentoxifylline was 0.01 nmol. The velocity of pentoxifylline formation at 5 μM lisofylline was determined in duplicate in microsomes prepared from 14 human livers for comparison with human liver microsomal protein concentration (up to 2 mg/ml) (data not shown).

In vitro kinetic parameters of microsomal lisofylline oxidation to pentoxifylline were determined using substrate concentrations ranging from 0.1 to 100 μM in microsomes prepared from four human livers. Results of a representative microsomal preparation, HL-142, are presented in Table 1. Each data point represents the mean value of duplicate incubation.

Results and Discussion

Metabolism of Lisofylline to Pentoxifylline in Human Liver Microsomes. Pentoxifylline formation by human liver microsomes was linear with respect to time of incubation (up to 10 min) and microsomal protein concentration (up to 2 mg/ml) (data not shown). In vitro kinetic parameters of microsomal lisofylline oxidation to pentoxifylline were determined using substrate concentrations ranging from 0.1 to 100 μM in microsomes prepared from four human livers. Results of a representative microsomal preparation, HL-142, are presented in Table 1.

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tbody>
<tr>
<td>Michaelis-Menten parameters for pentoxifylline formation in human liver microsomes</td>
</tr>
<tr>
<td>---</td>
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<tr>
<td><strong>K_m</strong> (μM)</td>
</tr>
<tr>
<td>HL-127</td>
</tr>
<tr>
<td>HL-135</td>
</tr>
<tr>
<td>HL-142</td>
</tr>
<tr>
<td>HL-146</td>
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<tr>
<td>Mean</td>
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<tr>
<td>SD</td>
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shown in fig. 1. The Eadie-Hofstee plot for pentoxifylline formation was biphasic, indicating the involvement of at least two isoforms of P450. Values of $K_m$ and $V_{\text{max}}$ determined in four human liver microsomes are listed in table 1. The mean ($\pm$ SD) values for $K_m$ and $V_{\text{max}}$ were $0.282 \pm 0.135 \mu M$ and $0.003 \pm 0.001 \text{nmol/min/mg protein}$, and for $K_m$ and $V_{\text{max}}$ were $158 \pm 42.6 \mu M$ and $0.928 \pm 0.308 \text{nmol/min/mg}$ protein, respectively.

Chemical Inhibition Studies. To identify the low apparent $K_m$ enzyme, a range of cytochrome P450 isozyme-selective inhibitors were incubated with 5 $\mu M$ lisofylline in five human liver microsome preparations (HL-127, 135, 139, 142, and 146). The results are shown in table 2. Furafylline and DDC inhibited pentoxifylline formation by 59% and 22%, respectively. No other compound caused significant inhibition of pentoxifylline formation. The combination of furafylline and DDC inhibited formation of pentoxifylline by 71% in the same five livers (table 2), which was not significantly different from the inhibition caused by furafylline alone.

The identity of the high apparent $K_m$ enzyme was probed in the same manner as the low apparent $K_m$ enzyme with 200 $\mu M$ lisofylline in HL-127 and HL-146 (data not shown). The maximum inhibition with any of the chemical inhibitors was 16%, observed with furafylline. Increasing the concentration of competitive inhibitors by $>10$-fold did not result in inhibition. ABT inhibited pentoxifylline formation by only 10%, and N-octylamine by 22%. There was no pentoxifylline formed when NADPH was omitted and none was formed by boiled microsomes in the presence of NADPH.

Activity Correlation Studies. The correlation between pentoxifylline formation (5 $\mu M$) with R-warfarin 6-hydroxylation (1 mM R-warfarin) as a probe of CYP1A2 activity and with chlorzoxazone 4-hydroxylation (500 $\mu M$ chlorzoxazone) as a probe of CYP2E1 activity was investigated.

### Table 2

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration (µM)</th>
<th>% of Control Activity</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HL-127</td>
<td>HL-135</td>
</tr>
<tr>
<td>Furafylline</td>
<td>30</td>
<td>43.9</td>
<td>51</td>
</tr>
<tr>
<td>Coumarin</td>
<td>8</td>
<td>87.8</td>
<td>94.1</td>
</tr>
<tr>
<td>Orphenadrine</td>
<td>100</td>
<td>122</td>
<td>129</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>400</td>
<td>114</td>
<td>99.8</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>20</td>
<td>109</td>
<td>84.6</td>
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<tr>
<td>S-mephenytoin</td>
<td>500</td>
<td>118</td>
<td>92.6</td>
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<tr>
<td>Quinidine</td>
<td>1</td>
<td>95.8</td>
<td>92.1</td>
</tr>
<tr>
<td>DDC</td>
<td>150</td>
<td>76.7</td>
<td>78.9</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>40</td>
<td>114</td>
<td>115</td>
</tr>
<tr>
<td>Furafylline + DDC</td>
<td>30/150</td>
<td>28.0</td>
<td>39.2</td>
</tr>
</tbody>
</table>

Values are mean of triplicate incubations.

DDC: Diethyldithiocarbamate.

a Not significantly different from furafylline alone, paired t-test, $p > 0.05$.

![Fig. 2. Correlation between pentoxifylline formation at 5 $\mu M$ lisofylline and A) CYP1A2 activity (R-warfarin 6-hydroxylation and B) CYP2E1 activity (chlorzoxazone 4-hydroxylation). C) CYP1A2 activity and CYP2E1 activity in microsomes prepared from 14 human livers.](image-url)
activity was assessed in microsomes prepared from 14 human livers (fig. 2). The pentoxifylline formation varied approximately 10-fold, from 0.0152 to 0.141 nmol/min/mg protein, among the 14 livers. A strong correlation ($r^2 = 0.870$, $p < 0.001$) was observed between pentoxifylline formation and R-warfarin 6-hydroxylation. There was no correlation between pentoxifylline formation and chlorzoxazone 4-hydroxylation ($r^2 = 0.143$, $p = 0.181$) or between R-warfarin 6-hydroxylation and chlorzoxazone 4-hydroxylation ($r^2 = 0.141$, $p = 0.183$).

**Metabolism in Human cDNA Expressed CYP1A2 and 2E1.** The formation of pentoxifylline from lisofylline in cDNA expressed CYP1A2 and CYP2E1 was determined to assess directly the formation of pentoxifylline by these enzymes. The velocities for pentoxifylline formation in cDNA expressed CYP1A2 were 0.987 and 4.73 nmol/min/mmol cytochrome P450 at 5 and 30 μM lisofylline, respectively. Pentoxifylline was not formed in cDNA expressed CYP2E1 at either 5 or 30 μM lisofylline. To reconcile the inhibition of pentoxifylline formation by DDC in human liver microsomes (which has generally been taken to indicate CYP2E1 inhibition) 150 μM DDC (preincubated as described for microsomal studies) was included in incubations with cDNA-expressed CYP1A2 at 5 μM lisofylline. DDC inhibited pentoxifylline formation by 85.7% in cDNA-expressed CYP1A2.

Taken together, these results indicate that CYP1A2 is the low apparent $K_m$ enzyme forming pentoxifylline from lisofylline in human liver microsomes. The high apparent $K_m$ enzyme appears not to be a cytochrome P450 2C19, as no specific or general chemical inhibitor of these enzymes resulted in significant inhibition. The enzyme responsible for this process is denatured by boiling and requires NADPH.

**References**


