IDENTIFICATION OF CYTOCHROME P-450 ISOFORMS RESPONSIBLE FOR C/S-TRAMADOL METABOLISM IN HUMAN LIVER MICROSOMES

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ABSTRACT:

The metabolism of cis-tramadol has been studied in human liver microsomes and in cDNA-expressed human cytochrome P-450 (CYP) isoforms. Human liver microsomes catalyzed the NADPH-dependent metabolism of tramadol to the two primary tramadol metabolites, namely, O-desmethyl-tramadol (metabolite M1) and N-desmethyl-tramadol (metabolite M2). In addition, tramadol was also metabolized to two minor secondary metabolites (each comprising <3.0% of total tramadol metabolism), namely, N,N-didesmethyl-tramadol (metabolite M3) and N,O-didesmethyl-tramadol (metabolite M5). Kinetic analysis revealed that multiple CYP enzymes were involved in the metabolism of tramadol to both M1 and M2. For the high-affinity enzymes involved in M1 and M2 formation, \( K_m \) values were 116 and 1021 \( \mu \)M, respectively. Subsequent reaction phenotyping studies were performed with a tramadol substrate concentration of 250 \( \mu \)M. In studies with characterized human liver microsomal preparations, good correlations were observed between tramadol metabolism to M1 and M2 and enzymatic markers of CYP2D6 and CYP2B6, respectively. Tramadol was metabolized to M1 by cDNA-expressed CYP2D6 and to M2 by CYP2B6 and CYP3A4. Tramadol metabolism in human liver microsomes to M1 and M2 was markedly inhibited by the CYP2D6 inhibitor quinidine and the CYP3A4 inhibitor troleandomycin, respectively. In summary, this study demonstrates that cis-tramadol can be metabolized to tramadol metabolites M1, M2, M3, and M5 in human liver microsomal preparations. By kinetic analysis and the results of the reaction phenotyping studies, tramadol metabolism in human liver is catalyzed by multiple CYP isoforms. Hepatic CYP2D6 appears to be primarily responsible for M1 formation, whereas M2 formation is catalyzed by CYP2B6 and CYP3A4.

Tramadol, (2-[[dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol), is a synthetic opioid analgesic of the aminocyclohexanol type that has two chiral centers (Paar et al., 1992). It is a centrally acting analgesic drug with an analgesic efficacy and a potency that ranges between weak opioids and morphine (Poulson et al., 1996). The biological activity of tramadol was initially attributed to predom- inantly \( \mu \)-receptor-mediated opioid analgesia. However, tramadol has only a low affinity for \( \mu \)-receptors of the central nervous system, being 6000 times lower than that of morphine. One metabolite of tramadol, namely, O-desmethyl-tramadol (metabolite M1\(^2\)), is known to have a higher affinity for opioid receptors than the parent drug. In contrast to other opioids, the analgesic action of tramadol is only partially inhibited by the opioid antagonist naloxone, which suggests an additional mechanism of action. Indeed, tramadol may act by both opioid and monoaminergic mechanisms, the latter involving inhibition of norepinephrine reuptake and stimulation of serotonin release (Collart et al., 1993a,b; Dayer et al., 1994, 1997; Poulsen et al., 1996).

The metabolism of tramadol has been studied in humans, rats, mice, Syrian hamsters, guinea pigs, rabbits, and dogs (Lintz et al., 1981). Following oral doses of \(^{14}\)C-labeled tramadol of either 1.06 or 1.25 mg/kg to two human volunteers, around 90% of the administered radioactivity was excreted in the urine. Analysis of 0- to 72-h urine samples revealed some unchanged tramadol (25 or 32% of total urinary radioactivity) and a number of tramadol metabolites. The primary metabolites (Fig. 1) of tramadol, namely, O-desmethyl-tram-adol (metabolite M1) and N-desmethyl-tramadol (metabolite M2) may be further metabolized to three additional secondary metabolites, namely, N,N-didesmethyl-tramadol (metabolite M3), N,N,O-tridesmethyl-tramadol (metabolite M4), and N,O-didesmethyl-tramadol (metabolite M5). The urine of the two subjects given \(^{14}\)C-labeled tramadol contained various amounts of these tramadol metabolites, together with conjugates (glucuronides and sulfates) of metabolites M1, M4, and M5 and some unidentified components.

The O-demethylation of tramadol to metabolite M1 is known to be catalyzed by human hepatic CYP2D6 (Paar et al., 1992, 1997; Poulsen et al., 1996). This cytochrome P-450 (CYP) isoform (Nelson et al., 1996) is expressed polymorphically in humans with some 93% of
Caucasians being phenotypically extensive metabolizers and the remainder being poor metabolizers of CYP2D6 probe substrates such as debrisoquine and sparteine (Dahl et al., 1995; Ingelman-Sundberg et al., 1995). A close relationship has been reported between tramadol O-demethylation and sparteine oxidation in human volunteers and tramadol may show a weaker analgesic effect on clinical pain in poor than in extensive metabolizers (Poulsen et al., 1996; Paar et al., 1997).

In the study of Lintz et al. (1981), a marked difference was observed between the two subjects examined in the relative amounts of M1 and M2 formed. Possibly one subject may have been CYP2D6 deficient (i.e., a poor metabolizer). In another study where a 50-mg oral dose of tramadol was given to 104 volunteers, mean values for M1 and M2 excretion in 24-h urines were 15 and 4% of the administered dose, respectively (Paar et al., 1997). Unlike tramadol O-demethylation, no correlation was observed between tramadol N-demethylation and sparteine oxidation in extensive metabolizers (Paar et al., 1997).

The in vitro metabolism of both (−)- and (+)-tramadol has been investigated in human liver microsomes with a substrate concentration range of 50 to 5000 μM (Paar et al., 1992). For O-demethylation to metabolite M1, the $K_{m}$ value for both isomers was determined to be 210 μM. While Eadie-Hofstee plots for M1 formation were monophasic, tramadol N-demethylation to metabolite M2 was stated to exhibit biphasic kinetics. Moreover, M1 formation, but not M2 formation, was competitively inhibited by quinidine with a reported $K_i$ value of 15 nM. Other studies have demonstrated that quinidine is a selective inhibitor of human hepatic CYP2D6 (Newton et al., 1995; Clarke, 1998; Pelkonen et al., 1998).

The objective of this study was to obtain more information on the CYP isoforms responsible for cis-tramadol metabolism in human liver. Investigations have been performed with human liver microsomes and cDNA-expressed CYP isoforms. The cis-tramadol metabolites studied were the two primary metabolites, namely, M1 and M2, together with two secondary metabolites, namely, M3 and M5 (Fig. 1).

**Experimental Procedures**

**Materials.** NADPH, cytochrome c, Tris, diethyldithiocarbamate, quinidine, sulfaphenazole, and troleandomycin were obtained from Sigma (Poole, Dorset,
Metabolism of Tramadol by Human Liver Microsomes. The NADPH-dependent metabolism of tramadol was studied in incubation mixtures containing 5 to 5000 μM tramadol (added in 5 μl of methanol), 10 mM MgCl₂, 2 mM EDTA, 80 mM phosphate buffer, pH 7.4, and 0.25 to 1.5 mg of microsomal protein in a final volume of 1 ml. After a 5-min preincubation at 37°C in a shaking water bath, the reaction was initiated by the addition of 1 mM NADPH. Incubations were performed for 3 to 30 min at 37°C and were terminated by the addition of 2 ml of ice-cold acetonitrile containing the [13C]- and deuterium-labeled internal standards for LC-MS-MS analysis (see below). Blank incubations contained all components except NADPH, which was added after the reaction was terminated with acetonitrile. For each tramadol substrate concentration, incubation time, and microsomal protein concentration incubations were conducted in duplicate with either a single or a duplicate blank (no NADPH) tube. Apart from the studies with the CYP1A2 mechanism-based inhibitor furafylline (Newton et al., 1995), where dimethyl sulfoxide (DMSO) was used, methanol was used as the solvent for these investigations to minimize solvent effects on individual CYP isoforms (Chauvet et al., 1998; Busby et al., 1999).

Tramadol metabolism to tramadol metabolites M1, M2, M3, and M5 was quantified by LC-MS-MS analysis. An HTS PAL autosampler (Preseal Ltd., Hitchin, Herts, UK) attached to an HP1100 series high-performance liquid chromatograph (Hewlett Packard Ltd., Bracknell, Berks, UK) were used to introduce samples into a Micromass Quattro LC triple quadrupole mass spectrometer (Micromass UK Ltd., Altrincham, Cheshire, UK) using an Electrospray Z-spray source. All incubations were terminated by the addition of two volumes of ice-cold acetonitrile containing 300 ng/ml of each of the [13C]- and deuterium-labeled tramadol metabolite M1, M2, M3, and M5 internal standards. In preliminary experiments cis-tramadol-[13C]-D₃ was also used as an internal standard. After vortex mixing and centrifugation at 5000g for 20 min at 4°C an aliquot of the supernatant was removed and appropriately diluted prior to analysis. For example, for all studies using a tramadol substrate concentration of 250 μM, samples were diluted with 1 volume of water and 8 volumes of 30% (v/v) acetonitrile in water. Chromatography of 20-μl aliquots of the diluted supernatants was performed with a 100 × 3.0 mm Kromasil C18 100A, 5-μm column (catalog no. 56105-078, Hypersil; Runcorn, Cheshire, UK) and isocratic elution with a mobile phase of 30% (v/v) acetonitrile in water, containing 0.1% (v/v) glacial acetic acid, at a flow rate of 0.3 ml/min and column temperature of 35°C. The eluate was passed directly into the triple quadrupole mass spectrometer operating in positive electrospray mode with probe and source block temperatures of 400 and 110°C, respectively, using nitrogen for desolvation and nebulization and a capillary voltage of 3.2 kV to initiate ionization. The compounds were selectively detected by multiple reaction monitoring using argon and quantitation was carried out against triplicate spiked aqueous standards at either four or six levels using the appropriate [13C]- and/or deuterium-labeled analog as internal standard for each analyte and a calibration curve produced for each metabolite. Table 1 shows the retention time, multiple reaction monitoring function, cone voltage, and collision energy for each compound analyzed.

Kinetics of Tramadol Metabolism. Kinetic data were analyzed by Michaelis-Menten and Eadie-Hofstee plots. Inspection of Eadie-Hofstee plots revealed that multiple enzymes were involved in the metabolism of tramadol to both tramadol metabolites M1 and M2. Kinetic analysis was performed by inspection of the Eadie-Hofstee plots and considering the data as biphasic kinetics with both high-affinity and low-affinity enzymes. For the high-affinity enzyme, Kₘ and Vₘₐₓ values were calculated directly from the Eadie-Hofstee plots. Additional calculations were performed to calculate the contribution of the high-affinity enzyme to the low-affinity enzyme kinetic plot. The following equation was used:

\[
\text{Calculated rate at a given substrate concentration} = \frac{V_{\text{max}} \cdot [S]}{K_{\text{m}} + [S]}
\]

The [13C]- and deuterium-labeled compounds were employed as internal standards for LC-MS-MS quantification of tramadol and tramadol metabolites.

UK) and furafylline and S(-)-mephentoin from Salford Ultrafine Chemicals and Research Ltd. (Manchester, UK). A reaction phenotyping kit (product no. H0500, version 5) containing 16 individual human liver microsomal preparations, characterized for total CYP content and a range of CYP isoform activity, was purchased from Xenotech LLC (Kansas City, KS) and stored at −80°C. Microsomes from human B-lymphoblastoid cells containing cDNA-expressed human CYP isoforms (GENTEST, Woburn, MA) were obtained from Cambridge Bioscience (Cambridge, UK) and were stored at −80°C. The samples of human B-lymphoblastoid cell microsomes comprised control cell microsomes (i.e., no transfected human cytochrome P-450 isoform cDNA but containing native CYP1A1 activity) and cell microsomes containing CYP1A2, CYP2A6 + OR [i.e., CYP2A6 cDNA plus human NADPH-cytochrome P-450 reductase (OR) cDNA], CYP2B6, CYP2C8 + OR, CYP2C9 + OR, CYP2C19, CYP2D6 + OR, CYP2E1 + OR, and CYP3A4 + OR. Samples of human liver (surplus to transplant operations) were obtained from Cambridge Bioscience (Cambridge, UK). Microsomes from human B-lymphoblastoid cell microsomes were prepared in a final volume of 1 ml of methanol, 10 mM MgCl₂, 2 mM EDTA, 80 mM phosphate buffer, pH 7.4, and 0.25 to 1.5 mg of microsomal protein in a final volume of 1 ml. After a 5-min preincubation at 37°C in a shaking water bath, the reaction was initiated by the addition of 1 mM NADPH. Incubations were performed for 3 to 30 min at 37°C and were terminated by the addition of 2 ml of ice-cold acetonitrile containing the [13C]- and deuterium-labeled internal standards for LC-MS-MS analysis (see below). Blank incubations contained all components except NADPH, which was added after the reaction was terminated with acetonitrile.
where \( V_{\text{max}} \) and \( K_m \) are the kinetic constants for the high-affinity enzyme and [S] is the substrate concentration. Calculated rates were derived for each substrate concentration used to calculate the low-affinity component. To derive corrected rates for the low-affinity component the following equation was used:

\[
\text{Low affinity component rate} = \frac{\text{Experimentally observed rate}}{1 + \frac{[S]}{K_m}}
\]

As an alternative approach estimates of \( K_a \) and \( V_{\text{max}} \) for both the high- and low-affinity enzymes were obtained with the following modified Michaelis-Menten equation (Clarke, 1998):

\[
y = \frac{V_{\text{max1}} \cdot [S]}{(K_m + [S])} + \frac{V_{\text{max2}} \cdot [S]}{(K_m + [S])}
\]

Inspection of the data revealed that the low-affinity component was not saturated over the range of tramadol substrate concentrations used. The following modified Michaelis-Menten equation (Clarke, 1998) was used:

\[
y = \frac{V_{\text{max1}} \cdot [S]}{(K_m + [S])} + \frac{V_{\text{max2}} \cdot [S]}{(K_m + [S])} + \frac{CL_{\text{int}} \cdot [S]}{K_m}
\]

In this equation \( CL_{\text{int}} \) represents the intrinsic clearance (i.e., \( V_{\text{max}}/K_m \)) of the low-affinity component.

**Tramadol Metabolism Correlation Analysis.** Incubation mixtures contained 250 \( \mu \)M tramadol (added in 5 \( \mu \)l of methanol), 10 mM MgCl\(_2\), 2 mM EDTA, 0.4 mg of microsomal protein, and 87 mM phosphate buffer, pH 7.4, in a final volume of 1 ml. After a 5-min preincubation at 37°C in a shaking water bath, the reaction was initiated by addition of 1 mM (final concentration) NADPH. Incubations were conducted in triplicate with a single blank (no NADPH) for each of the 16 preparations of characterized human liver microsomes and were terminated after 5 min by addition of 2 ml of ice-cold acetonitrile containing the \(^{13}\)C- and deuterium-labeled tramadol metabolite M1, M2, M3, and M5 internal standards. Incubations were processed for LC-MS-MS analysis as described above.

**Tramadol Metabolism by cDNA-Expressed CYP Isoforms.** A 100 mM stock solution of tramadol in methanol was diluted to 333 mM with 100 mM phosphate buffer, pH 7.4. Incubation mixtures contained 250 \( \mu \)M tramadol (added as 187.5 \( \mu \)l of 333 \( \mu \)M tramadol), 10 mM MgCl\(_2\), 2 mM EDTA, 1 mM NADPH, and 80 mM phosphate buffer in a final volume of 0.25 ml. After 5-min preincubination in a 37°C water bath, the reaction was initiated by adding 0.25 mg of B-lymphoblastoid cell microsomal protein with gentle mixing. The incubations were conducted in duplicate with a single blank (no microsomes) for each CYP isoform preparation. Reactions were terminated after either 5 or 30 min with 0.5 ml of ice-cold acetonitrile containing the \(^{13}\)C- and deuterium-labeled tramadol metabolite M1, M2, M3, and M5 internal standards. Incubations were processed for LC-MS-MS analysis as described above.

**Tramadol Metabolism Inhibition Studies.** For the mechanism-based inhibitors requiring preincubation with NADPH, incubation mixtures contained 10 mM MgCl\(_2\), 2 mM EDTA, 1 mM NADPH, 85 mM phosphate buffer, pH 7.4, 0.5 mg of pool II microsomal protein, and either 5 to 50 \( \mu \)M furafylline, 5 to 100 \( \mu \)M diethyldithiocarbamate, or 5 to 100 \( \mu \)M tromethamycin in a volume of 0.945 ml. The inhibitors were added in either methanol (5 \( \mu \)l/tube) or DMSO (furafylline only, 5 \( \mu \)l/tube) and the concentrations refer to the final concentrations in a 1-ml incubation. Following a 30-min preincubation at 37°C in a shaking water bath, 250 \( \mu \)M tramadol (added in 5 \( \mu \)l of methanol) and 1 mM NADPH (final concentration 2 mM) were added. Incubations were performed for 5 min at 37°C and were terminated by the addition of 2 ml of ice-cold acetonitrile containing the \(^{13}\)C- and deuterium-labeled tramadol metabolite M1, M2, M3, and M5 internal standards. All test incubations were performed in either triplicate (no inhibitor added controls, but with either 5 \( \mu \)l of methanol or 5 \( \mu \)l of DMSO) or duplicate (for each concentration of each inhibitor), with single blank tubes where tramadol was added after the ice-cold acetonitrile. Incubations were processed for LC-MS-MS analysis as described above.

For the compounds not requiring extensive preincubation with NADPH, incubation mixtures contained 10 mM MgCl\(_2\), 2 mM EDTA, 1 mM NADPH, 0.5 mg of pool II microsomal protein, 85 mM phosphate buffer, pH 7.4, and either 2 to 50 \( \mu \)M sulfaphenazole, 2 to 20 \( \mu \)M quinidine, or 50 to 500 \( \mu \)M S-mephenytoin in a volume of 0.995 ml. All the inhibitors were added in methanol (5 \( \mu \)l/tube) and the concentrations refer to the final concentrations in a 1-ml incubation. Following a 10-min preincubination at 37°C in a shaking water bath, the reaction was initiated by the addition of 250 \( \mu \)M tramadol (added in 5 \( \mu \)l of methanol). Incubations were performed for 5 min at 37°C and were terminated by the addition of 2 ml of ice-cold acetonitrile containing the \(^{13}\)C- and deuterium-labeled tramadol metabolite M1, M2, M3, and M5 internal standards. All test incubations were performed in either triplicate (no inhibitor added but containing 5 \( \mu \)l of methanol/tube) or duplicate (for each concentration of each inhibitor), with single blank tubes where tramadol was added after the ice-cold acetonitrile. Incubations were processed for LC-MS-MS analysis as described above.

**Results**

**Metabolism of Tramadol by Human Liver Microsomes.** Tramadol was metabolized by human liver microsomes in the presence of NADPH to all four available tramadol metabolites. While M1 and M2 were major metabolites of tramadol in human liver microsomes, M3 and M5 represented only minor metabolites. Some representative data for tramadol metabolism to metabolites M1, M2, M3, and M5 are shown in Table 2. Under the incubation conditions selected, tramadol metabolism to either metabolite M3 or metabolite M5 was always 3.0% of total tramadol metabolism (i.e., the sum of formation of M1, M2, M3, and M5) from Table 2. Under the incubation conditions selected, tramadol metabolism to M1 and M2 was found to

**TABLE 2**

<table>
<thead>
<tr>
<th>Microsomal Preparation</th>
<th>Tramadol Substrate Concentration</th>
<th>Tramadol Metabolism (pmol/min/mg protein)(^a)</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M5</th>
<th>Total(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool I</td>
<td>20</td>
<td>22.1 (38.3(^c))</td>
<td>32.2 (57.5)</td>
<td>0.7 (1.2)</td>
<td>1.7 (3.0)</td>
<td>57.7 (100)</td>
<td></td>
</tr>
<tr>
<td>Pool I</td>
<td>200</td>
<td>93.0 (24.4)</td>
<td>278.3 (73.0)</td>
<td>5.0 (1.3)</td>
<td>4.9 (1.3)</td>
<td>381.2 (100)</td>
<td></td>
</tr>
<tr>
<td>Pool I</td>
<td>500</td>
<td>148.0 (28.5)</td>
<td>367.0 (70.8)</td>
<td>3.3 (0.6)</td>
<td>0.3 (0.1)</td>
<td>518.6 (100)</td>
<td></td>
</tr>
<tr>
<td>Pool II</td>
<td>500</td>
<td>87.3 (40.3)</td>
<td>128.7 (59.4)</td>
<td>&lt;0.1 (0)</td>
<td>0.7 (0.3)</td>
<td>216.7 (100)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Duplicate test and single blank incubations were performed with each tramadol substrate concentration and each human liver microsomal preparation. Incubations were performed with 0.4 to 1.0 mg of microsomal protein for 5 or 10 min.

\(^b\)Sum of tramadol metabolism to metabolites M1, M2, M3, and M5.

\(^c\)Numbers in parentheses are percentages of total tramadol metabolism.
be essentially linear (data not shown) with respect to incubation time (up to at least 6 min) and protein concentration (up to at least 1.0 mg of protein/ml). In contrast, tramadol metabolism to the two secondary metabolites, namely, M3 and M5, exhibited poor linearity (data not shown). The linearity of tramadol metabolism to metabolites M1 and M2 was also investigated with the pool II microsomal preparation. The formation of M1 and M2, but not M3 and M5, was found to be linear with respect to incubation time and microsomal protein concentration, except that this preparation was less active than the pool I preparation (data not shown). The total CYP content of the pool I and II microsomal preparations was determined to be 0.72 and 0.51 pmol/mg of protein, respectively, whereas NADPH-cytochrome c reductase activities were determined to be 356 and 348 nmol/min/mg of protein, respectively.

Kinetics of Tramadol Metabolism in Human Liver Microsomes.

The kinetics of the NADPH-dependent metabolism of 5 to 5000 µM tramadol to metabolites M1 and M2 was examined with both the pool I and pool II human liver microsomal preparations. Because both metabolites M3 and M5 each constituted ≤3.0% of total tramadol metabolism in human liver microsomes, it was not considered necessary to investigate the kinetics of formation of these two metabolites. Pooled, rather than individual, liver microsomal preparations were used for these studies, because the aim was to identify average values to select a suitable substrate concentration for the reaction phenotyping studies, rather than to evaluate any interindividual variability in K_m values for M1 and M2 formation. With both liver microsome pools, M1 and M2 were detected at all tramadol substrate concentrations examined (Fig. 2). Figure 2 also demonstrates that while M1 is the major metabolite formed at low tramadol substrate concentrations, M2 formation predominates at high substrate concentrations. In keeping with the higher total CYP content, the pool I liver microsome preparation was more active than the pool II microsomal preparation in catalyzing M1 and M2 formation.

The kinetics of tramadol metabolism to M1 and M2 was examined by both Eadie-Hofstee and Michaelis-Menten plots. With both pools of human liver microsomes, analysis of Eadie-Hofstee plots revealed that multiple enzymes were involved in the metabolism of tramadol to metabolite M1 (Fig. 3, A and C) and to metabolite M2 (Fig. 3, B and D). However, Eadie-Hofstee plots of the corrected low-affinity enzyme data were not linear and hence no K_m and V_max values could be calculated. As an alternative approach a modified Michaelis-Menten equation (see Experimental Procedures) was used to try to obtain estimates of K_m and V_max. However, inspection of the plots obtained indicated that the low-affinity enzymes involved in M1 and M2 formation were not saturated over the range of tramadol substrate concentrations used. For the low-affinity enzymes involved in M1 and M2 formation CL_int values were calculated (see Experimental Procedures).

The results of the kinetic analysis of tramadol metabolism to M1 and M2 by the two pools of human liver microsomes examined are shown in Table 3. For the high-affinity enzyme involved in M1 formation K_m values of 105 and 126 µM were observed with the pool I and II liver microsomal preparations, respectively. These K_m values are similar to the value of 210 µM reported by Paar et al. (1992) for a preparation of human liver microsomes obtained from a single donor. Overall, for both liver microsome pools, mean K_m values of 116 and 1021 µM were calculated for the high-affinity enzymes responsible for M1 and M2 formation, respectively. For the high- and low-affinity enzymes involved in M1 and M2 formation, CL_int values were 1.47 and 0.048 µl/min/mg of protein and 0.69 and 0.112 µl/min/mg of protein, respectively (Table 3).

A tramadol substrate concentration of 250 µM was selected for the reaction phenotyping studies described below. This substrate concentration was selected as being approximately 2 times the K_m value for M1 formation by the high-affinity enzyme (Table 3).

Tramadol Metabolism Correlations Analysis. The metabolism of 5 to 5000 µM tramadol to M1 and M2 was studied with the pool I (A) and II (B) human liver microsomal preparations. Incubations were performed with 0.5 mg of microsomal protein for 5 min. Results are presented as the mean of a duplicate test and a duplicate blank incubation.

The metabolism of 5 to 5000 µM tramadol to M1 and M2 was examined with a characterized panel of 16 human liver microsomal preparations. Although tramadol metabolism to the secondary metabolites M3 and M5 was not necessarily linear under the incubation conditions selected, the levels of these two minor tramadol metabolites were also quantified in the incubation extracts. Tramadol was metabolized to M1 and M2 by all 16 human liver microsomal preparations examined (Fig. 4A). Compared with M1 and M2 formation, much smaller amounts of the secondary metabolites M3 and M5 were observed in 14 of the 16 human liver microsomal preparations examined (Fig. 4B).

The rates of tramadol metabolism to M1, M2, M3, and M5 were correlated with data for total CYP content and a range of CYP isoform enzyme activities (Table 4). For tramadol metabolism to M1 the best correlation (r^2 = 0.676) was observed with the CYP2D6 isoform dextromethorphan O-demethylase activity (Table 4). A lower correlation (r^2 = 0.380) was observed with the CYP2B6 marker 5-methoxy-7-ethylindole N-demethylase. In contrast, for M2 formation the best correlation (r^2 = 0.748) was observed with the S-mephenytoin N-demethylase activity. Lower correlations were observed with the CYP2C8 marker (r^2 = 0.454) taxol 6β-hydroxylase and the CYP3A4 marker (r^2 = 0.290) testosterone 6β-hydroxylase. For both M1 and...
M2 formation and total CYP content and CYP isoform markers for CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2E1, and CYP4A9/11, \( r^2 \) values of 0.0004 to 0.217 were observed (Table 4). These enzyme activities comprised 7-ethoxyresorufin \( O \)-deethylase, coumarin 7-hydroxylase, diclofenac 4'-hydroxylase, \( S \)-mephenytoin 4'-hydroxylase, chlorzoxazone 6-hydroxylase, and lauric acid 12-hydroxylase. For the secondary tramadol metabolites M3 and M5 some apparent correlations (\( r^2 = 0.357–0.601 \)) were observed with \( S \)-mephenytoin \( N \)-demethylase and taxol 6\( \alpha \)-hydroxylase activities (Table 4).

**Metabolism of Tramadol by cDNA-Expressed CYP Isoforms.** The metabolism of 250 \( \mu M \) tramadol by B-lymphoblastoid cell microsomes containing cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, together with microsomes from control cells (which contain native CYP1A1), was studied. Only trace amounts of tramadol metabolites were observed in incubations with the control cell microsomes (data not shown) and with the cDNA-expressed CYP2A6, CYP2C8, and CYP2E1 preparations (Fig. 5). While tramadol metabolism to M1 was greatest with the cDNA-expressed CYP2D6 preparation, M2 formation was greatest with the cDNA-expressed CYP2B6 preparation (Fig. 5A). Tramadol was also metabolized to M1 by the CYP2B6 preparation and to M2 by the CYP2D6 and CYP3A4 preparations. Only low rates of tramadol metabolism to either M1 or M2 were observed with the cDNA-expressed CYP1A2, CYP2C9, and CYP2C19 preparations (Fig. 5A). Low rates of tramadol metabolism to M3 were observed with the CYP2B6 and CYP3A4 preparations, with a low rate of M5 formation being catalyzed by the CYP2D6 preparation (Fig. 5B).

**Inhibition of Tramadol Metabolism.** The effect of some human CYP isoform inhibitors and one CYP isoform substrate (\( S \)-mephenytoin) on the metabolism of 250 \( \mu M \) tramadol to metabolites M1 and M2 in human liver microsomes was studied. Because of the low rates of formation of M3 and M5, it was not possible to evaluate the effects of the test compounds on these tramadol secondary metabolites. For the mechanism-based inhibitors (Newton et al., 1995) furafylline (CYP1A2), diethyldithiocarbamate (CYP2E1), and troleandomycin...
concentrations of these two inhibitors (Fig. 6A). The addition of 2 to 20 μM quinidine markedly inhibited tramadol metabolism to M1 to 14 to 23% of control (Fig. 6C). In contrast, neither 2 to 50 μM sulfaphenazole nor 50 to 500 μM S-mephenytoin had any marked effect on M1 formation (Fig. 6C).

Tramadol metabolism to M2 was inhibited to 33 to 44% of control by 5 to 100 μM troleandomycin (Fig. 6B). In contrast, while 5 to 50 μM furafylline had no effect on M2 formation, 5 to 100 μM diethyldithiocarbamate resulted in some inhibition of high concentrations. While 2 to 50 μM sulfaphenazole inhibited M2 formation to some extent at high concentrations, neither 2 to 20 μM quinidine nor 50 to 500 μM S-mephenytoin had any marked effect on M2 formation (Fig. 6D).

Discussion

In this study the metabolism of cis-tramadol by human hepatic microsomal fractions and cDNA-expressed human hepatic CYP isoforms has been investigated. The formation of the major tramadol metabolites M1 and M2 was observed in two preparations of pooled liver microsomes and in 16 individual preparations. In addition, two minor secondary metabolites (each ≤3.0% of total metabolism), namely, M3 and M5 were also observed in the majority of the human liver microsomal preparations examined. Tramadol can thus be metabolized in human liver by either single or multiple O- and N-demethylations (Fig. 1).

For both M1 and M2 formation in human liver microsomes, biphasic Eadie-Hofstee plots were obtained, indicating the participation of more than one CYP isoform in these pathways of tramadol metabolism. The kinetic data were considered as biphasic plots with both high-affinity and low-affinity enzymes. Over a substrate concentration range of 5 to 5000 μM mean K_m values of 116 and 1021 μM were obtained for the high-affinity enzymes responsible for M1 and M2 formation, respectively (Table 3). While the mean K_m value of 116 μM for M1 formation is similar to the value of 210 μM reported by Paar et al. (1992) for metabolism of both (-) - and (+)-tramadol to M1, these studies only reported monophasic kinetics. However, in agreement with the present study, Paar et al. (1992) did observe biphasic kinetics for M2 formation although no K_m values were reported. In the present study, the lower K_m value for the high-affinity component of M1 formation compared with M2 formation (i.e., 116 compared with 1021 μM) is in agreement with the observation that in most subjects larger quantities of M1 are formed after oral administration of tramadol (Lintz et al., 1981; Paar et al., 1997).

To identify the CYP isoform responsible for tramadol metabolism,
“reaction phenotyping” was performed by correlation analysis with a panel of characterized microsomal preparations, chemical inhibition, and the use of cDNA-expressed human CYP isoforms. A cis-tramadol substrate concentration of 250 μM was selected as being approximately 2 times the $K_{\text{m}}$ for the high-affinity enzyme responsible for M1 formation.

The metabolism of cis-tramadol was examined in a panel of 16 characterized human liver microsomal preparations. Good correlations were obtained between M1 formation and dextromethorphan O-demethylase ($r^2 = 0.676$) and between M2 formation and S-mephenytoin N-demethylase ($r^2 = 0.748$). These two enzyme activities are considered to be markers for CYP2D6 and CYP2B6, respectively, in human liver microsomes (Heyn et al., 1996; Parkinson, 1996; Clarke, 1998; Pelkonen et al., 1998). Some lower correlations between either M1 or M2 formation and enzymatic markers for CYP2C8 and CYP3A4 were also observed (Table 4).

The conclusions of the correlation analysis study are supported by the results of the chemical inhibition studies. Previous studies have demonstrated that furafylline, sulfaphenazole, S-mephenytoin, quinidine, diethyldithiocarbamate, and troleandomycin may be considered either inhibitors or substrates of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4, respectively (Newton et al., 1995; Ono et al., 1996; Parkinson, 1996; Clarke, 1998; Pelkonen et al., 1998; Rodrigues, 1999). Tramadol metabolism to M1 was inhibited to 14 to 23% of control by the CYP2D6 inhibitor quinidine, whereas M2 formation was inhibited to 33 to 44% of control by the CYP3A4 mechanism-based inhibitor troleandomycin (Fig. 6, B and C). In contrast, the inhibitors or substrate of CYP1A2, CYP2C9, CYP2C19, and CYP2E1 studied had no marked effect on tramadol metabolism to either M1 or M2. The effect of a chemical inhibitor of CYP2B6 was not evaluated in this study, although S-mephenytoin, which is metabolized to different products by CYP2B6 and CYP2C19 (Relling et al., 1989; Heyn et al., 1996; Parkinson, 1996; Clarke, 1998; Pelkonen et al., 1998), had no marked effect on either M1 or M2 formation. The lack of a marked effect of S-mephenytoin on CYP2B6-catalyzed cis-tramadol metabolism to M2 in this study is probably attributable to...
the \( K_m \) value for CYP2B6-catalyzed \( S \)-mephenytoin \( N \)-demethylase. Other studies have demonstrated that while the \( K_m \) value for CYP2C19-catalyzed \( S \)-mephenytoin 4'-hydroxylase in human liver microsomes is around 60 to 65 \( \mu \)M, the \( K_m \) value for CYP2B6-catalyzed \( S \)-mephenytoin \( N \)-demethylase has been reported to be 805 \( \mu \)M (Relling et al., 1989; Heyn et al., 1996; Pelkonen et al., 1998). Thus, over the 50 to 500 \( \mu \)M concentration range studied, \( S \)-mephenytoin would not have been expected to have a marked effect on CYP2B6-catalyzed \( cis \)-tramadol metabolism to M2.

\( cis \)-Tramadol was metabolized to M1 and M2 by some of the cDNA-expressed human CYP isoforms examined. CYP2D6 was the major CYP isoform catalyzing M1 formation, whereas CYP2B6 and CYP3A4 were the major isoforms catalyzing M2 formation (Fig. 5). In contrast, cDNA-expressed CYP1A2, CYP2C9, and CYP2C19 only catalyzed low rates of M1 and M2 formation. For M2 formation the CYP2B6 preparation exhibited a higher specific activity than the CYP3A4 preparation. However, the levels of CYP3A4A in human liver microsomes have been reported to range from 44 to 250 pmol/mg of protein, whereas those for CYP2B6 have been reported to range from only 1 to 39 pmol/mg of protein (Lecoeur et al., 1994; Shimada et al., 1994; Imaoka et al., 1996; Rodrigues, 1999). Thus, assuming these two CYP isoforms have similar affinities (i.e., \( K_m \) values) for tramadol, M2 formation may be predominantly catalyzed by CYP3A4 in human liver microsomes.

This study demonstrates that multiple CYP isoforms are responsible for \( cis \)-tramadol metabolism in human liver. Clearly, tramadol metabolism to tramadol metabolite M1 is primarily catalyzed by CYP2D6 and this finding is in agreement with other in vivo and in vitro studies on tramadol metabolism and the effect of quinidine inhibition (Paar et al., 1992, 1997; Dayer et al., 1994; Poulsen et al., 1996). From the correlation analysis and cDNA-expressed CYP isoform data, CYP2B6 may have a minor role in M1 formation in human liver with very low levels of activity being catalyzed by CYP1A2, CYP2C9, and CYP2C19. While CYP2D6 appears to be the high-affinity enzyme catalyzing M1 formation in human liver microsomes, additional studies would be required to identify the low-affinity enzyme(s) responsible for M1 formation.

Unlike M1, the major CYP isoforms involved in M2 formation appear to be CYP2B6 and CYP3A4. Additional kinetic studies would be required to identify the high- and low-affinity enzymes catalyzing M2 formation in human liver microsomes. From the present data CYP2B6 may constitute a high-affinity enzyme, whereas the high levels of CYP3A4A in human liver (Lecoeur et al., 1994; Shimada et al., 1994; Imaoka et al., 1996; Rodrigues, 1999) would imply that this CYP isoform would also contribute to M2 formation, particularly in subjects with either low or absent levels of CYP2B6 (Mimura et al., 1993; Code et al., 1997; Shimada et al., 1997). Based on the cDNA-expressed CYP isoform data, CYP2D6 may have a minor role in M2 formation, with very low levels of activity being catalyzed by CYP1A2, CYP2C9, and CYP2C19. While some correlation \((r^2 = 0.454)\) was observed between CYP2C8-catalyzed taxol 6a-hydroxylase activity and M2 formation, this finding was not supported by

**Fig. 6.** Inhibition of tramadol metabolism to tramadol metabolites M1 and M2 in human liver microsomes.

The effects of the mechanism-based inhibitors (A and B) 5 to 50 \( \mu \)M furafylline (●), 5 to 100 \( \mu \)M diethylthiocarbamate (▼), and 5 to 100 \( \mu \)M troleandomycin (◆), together with (C and D) 2 to 50 \( \mu \)M sulfaphenazole (▲), 50 to 500 \( \mu \)M S-mephenytoin (■), and 2 to 20 \( \mu \)M quinidine (◆) were evaluated on the metabolism of 250 \( \mu \)M tramadol to M1 (A and C) and M2 (B and D) in human liver microsomes. Incubations were performed with 0.5 mg of pool II microsomal protein for 5 min. Each point represents the mean of a duplicate test and a single blank incubation.
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cDNA-expressed CYP isoform studies where only a very low rate of M2 formation was observed with the CYP2C8 preparation.

Some preliminary information was also obtained on the CYP isoforms involved in M3 and M5 formation. However, only tentative conclusions can be drawn from the present data because M3 formation is dependent on the amount of M2 produced and M5 formation is dependent on the production of both M1 and M2. In addition, under the incubation conditions used M3 and M5 formation was not necessarily linear with respect to incubation time and microsomal protein concentration. Based on the observations that CYP2D6 primarily catalyzes tramadol O-demethylation, whereas CYP2B6 and CYP3A4 primarily catalyze N-demethylation, CYP2D6 would be expected to participate in M5 formation from M2. In addition, CYP2B6 and CYP3A4 would be expected to participate in M1 metabolism to M5 and M2 metabolism to M3. Additional studies, including using M1 and M2 as substrates, would be required to fully elucidate the CYP isoforms involved in M3 and M5 formation.

In summary, cts-tramadol can be metabolized to tramadol metabolites M1, M2, M3, and M5 in human liver microsomal preparations. While M1 and M2 constitute major primary metabolites, M3 and M5 are only minor secondary metabolites. By kinetic analysis and the results of the reaction phenotyping studies, tramadol metabolism in human liver is catalyzed by multiple CYP isoforms. While CYP2D6 is primarily responsible for M1 formation, M2 formation is catalyzed by CYP2B6 and CYP3A4.

References
Ono S, Hatanaka T, Hotta H, Satoh T, Gonzalez FJ and Tsutsui M (1996) Specificity of substrate demethylation, CYP2D6 would be expected to participate in the formation of tramadol metabolites M1, M2, M3, and M5 in human liver microsomal preparations.