Zopiclone is a γ-aminobutyric acid receptor agonist that is widely prescribed for its hypnotic properties (Noble et al., 1998) in insomniac patients. As benzodiazepines agents, the duration of its pharmacological effect and the occurrence of side effects, such as morning hypnotic residual effects (Allan et al., 1991), are mainly dependent on its biological half-life and clearance.

In humans, zopiclone elimination is mainly dependent on its hepatic clearance because only 5% of the drug is excreted unchanged in the urine (Noble et al., 1998). Indeed, zopiclone is extensively metabolized by the human liver into two major metabolites (Fig. 1): N-desmethyl-zopiclone (ND-Z) and N-oxide-zopiclone (NO-Z) with the following $K_m$ and $V_m$ of 78 ± 5 and 84 ± 19 μM, 45 ± 1 and 54 ± 5 pmol/min/mg for ND-Z and NO-Z generation, respectively. Ketoconazole (CYP3A inhibitor) inhibited ~40% of the generation of both metabolites, sulfaphenazole (CYP2C inhibitor) inhibited the formation of ND-Z, whereas α-naphthoflavone (CYP1A), quinidine (CYP2D6), and chlorzoxazone (CYP2E1) did not affect zopiclone metabolism. The generation of ND-Z and NO-Z were highly correlated to testosterone 6β-hydroxylation (CYP3A activity, $r = 0.95$ and 0.92, respectively; $p = .0001$), and ND-Z was highly correlated to CYP2C8 activity (paclitaxel 6α-hydroxylase; $r = 0.76$, $p = .004$). Recombinant CYP2C8 had the highest enzymatic activity toward zopiclone metabolism into both its metabolites, followed by CYP2C9 and 3A4. CYP3A4 is the major enzyme involved in zopiclone metabolism in vitro, and CYP2C8 contributes significantly to ND-Z formation.

**Materials and Methods**

**Drugs, Chemicals, and Reagents.** Zopiclone, NO-Z, and ND-Z were kindly provided by Rhône-Poulenc Rorer (Antony, France) and ketoconazole by Jansen (Beerse, Belgium). Hydroquinidine was purchased from Fluka (Buchs, Switzerland), and sulfaphenazole, quinidine, chlorzoxazone, and α-naphthoflavone were obtained from Sigma Chemical Co. (St. Louis, MO). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and NADP were purchased from Boehringer Mannheim (Meylan, France); reagents for protein assays were obtained from Pierce Chemical Co. (Beitbergen, the Netherlands). All the other reagents and solvents were of the highest grade commercially available.

**Human Liver Microsomes.** Human liver microsomes from 12 different donors were provided by Gentest (Woburn, MA). Additional microsomes were prepared from liver samples of 10 human donors, collected, and stored as described previously (Becquemont et al., 1998).

**Yeast-Expressed Recombinant Human CYP (rH-CYP) Enzymes.** Human CYP1A2, 2C9, 2C19, 2C18, 2C9, 2D6, 2E1, and 3A4 were cloned and expressed in yeast strains that overexpress endogenous NADPH-P-450 reductase, as described previously (Gautier et al., 1996). Microsomes from the different yeast cultures were prepared by mechanical lysis, followed by differential ultracentrifugation (Renaud et al., 1990; Gautier et al., 1996).

**Proteins and CYP Concentration.** Human liver and yeast-expressed CYP microsomal concentrations were measured by a spectrophotometric method as described by Schoene et al. (1972). Total protein concentration was assayed by the bicinchoninic acid method (Pierce Chemical Co.) according to the supplier’s recommendation and using serum albumin as the standard.

**Quantification of CYP Activities.** CYP3A, CYP2C9, and CYP2D6 enzymatic activities (testosterone 6β-hydroxylation, diclofenac 4′-hydroxylation, and dextromethorphan O-demethylation) for the 22 liver samples were performed as described previously (Langouët et al., 1995; Funck-Brentano et al., 1998).
NaH₂PO₄ (70 mM, pH 8) buffer. After the addition of 50 U/ml glucose 6-phosphate dehydrogenase and stopped 60 min later on ice and by adding 500 μl of the NADPH-generating system consisting of 0.15 mM NADP, 2.5 mM glucose 6-phosphate, and 1.7 U/ml glucose 6-phosphate dehydrogenase. After 5-min incubation was carried out at 37°C in Tris-EDTA buffer in the presence of an NADPH-generating system and 1 mg of human liver microsomes or 100 pmol of the different rH-CYP isoforms in a final volume of 1 ml. Zopiclone cytochrome P450 content was determined previously by the manufacturer on the 12 samples provided from Gentest.

**Zopiclone Metabolism.** The kinetics of zopiclone oxidation and demethylation were studied in the presence of 1 mg of human liver microsomes or 100 pmol of the different rH-CYP isoforms in a final volume of 1 ml. Zopiclone was used at eight different concentrations ranging from 5 to 400 μM. Each incubation was carried out at 37°C in Tris-EDTA buffer in the presence of a NADPH-generating system consisting of 0.15 mM NADP, 2.5 mM glucose 6-phosphate, and 1.7 U/ml glucose 6-phosphate dehydrogenase. After 5-min preincubation, the reaction was started by adding the glucose 6-phosphate dehydrogenase and stopped 60 min later on ice and by adding 500 μl of NaH₂PO₄ (70 mM, pH 8) buffer. After the addition of 50 μl of the internal standard (hydroquinidine, 1 mM in methanol) and 2 ml of CH₂Cl₂, the preparation was mixed for 15 min and centrifuged during 5 min at 3000 g to remove the protein pellet. The organic phase was dried and dissolved in 200 μl of the HPLC mobile phase. HPLC analysis was performed on a 4.6×250-mm Symmetry C18 column (Waters, Milford, CT). Fluorescence detection was performed with an excitation wavelength of 300 nm and an emission wavelength of 470 nm. The isocratic mobile phase, consisting of 50 mM NaH₂PO₄ (pH 3.7) and acetonitrile 80:20 (v/v), was maintained at 1 ml/min during 30 min. The quantification limit of the method was 10 nM for ND-Z and 30 nM for NO-Z with an intraday coefficient of variation varying from 11 to 7%.

**Correlation studies with the 22 human microsomal samples were performed with an excitation wavelength of 300 nm and an emission wavelength of 470 nm.** The isocratic mobile phase, consisting of 50 mM NaH₂PO₄ (pH 3.7) and acetonitrile 80:20 (v/v), was maintained at 1 ml/min during 30 min. The quantification limit of the method was 10 nM for ND-Z and 30 nM for NO-Z with an intraday coefficient of variation varying from 11 to 7%.

**NO-Z and ND-Z formation rates were shown to be linear with time up to 60 min and with human microsomal protein and rH-CYP concentrations up to 2 mg/ml and 100 pmol/ml, respectively.**

Inhibition studies of zopiclone metabolism in human liver microsomes were performed in triplicate in the presence of a single zopiclone concentration of 50 μM and a single concentration of sulfaphenazole (10 μM), quinidine (10 μM), chloroxazone (100 μM), α-naphthoflavone (10 μM), or ketoconazole (0.5 μM). These experiments were performed on three different donor samples.

**Correlation studies with the 22 human microsomal samples were performed in duplicate under the same conditions at two zopiclone concentrations of 25 and 200 μM.**

**Determination of the CYP isoforms involved in zopiclone metabolism in the presence of the different rH-CYP isoforms (100 pmol) was performed in quadruplicate under the same conditions at a single zopiclone concentration of 50 μM.**

**Data Analysis.** K<sub>m</sub>, the apparent affinity constant, and V<sub>max</sub>, the maximum initial enzyme velocity, were initially evaluated by graphical examination of Eadie-Hofstee plots. These values were taken as initial parameters for the estimation of the Michaelis-Menten parameters and S.E.s by nonlinear least-squares regression curve fitting as described previously (Funck-Brentano et al., 1997; Becquemont et al., 1998).

To extrapolate the results of zopiclone metabolism obtained with rH-CYP to human liver microsomes, we determined the product of the turnover numbers obtained from each rH-CYP by the specific contents in human liver microsomes of each CYP isofrom (Becquemont et al., 1998). This P-450 content was estimated based on available data (Guengerich and Turvy, 1991; Shimada et al., 1994; Bello et al., 1996; Gautier et al., 1996) as being in the range of 69 pmol/mg for CYP1A2, 150 pmol/mg for CYP3A4, 90 pmol/mg for CYP2C9, 35 pmol/mg for CYP2E1, 20 pmol/mg for CYP2D6, and 10 pmol/mg for CYP2C8, CYP2C18, and CYP2C19. Results are presented as mean ± S.D.

**Results**

**Zopiclone Metabolism Kinetic Constants in Human Liver Microsomes.** We observed that zopiclone was metabolized into NO-Z and ND-Z in all of the liver microsomes from the 22 different human donors. There was a 45- and 30-fold extent variability in ND-Z and NO-Z generation rate, respectively, from one donor to another. We determined the enzymatic kinetics of zopiclone metabolism in two liver samples that were chosen among the 22 liver samples for their predetermined CYP3A activity. One liver had an intermediate CYP3A activity. One liver had an intermediate CYP3A activity. The other showed the lowest CYP3A activity. Enzymatic constants are presented in Table 1 and illustrated in Fig. 2. These two liver samples were found to have medium and very low turnover numbers toward the generation of both zopiclone metabolites (Fig. 3). Intrinsic clearance (V<sub>c</sub>/K<sub>m</sub>) of ND-Z and NO-Z were, respectively, 10- and 3-fold lower in the liver with the lowest CYP3A activity compared with the liver with medium CYP3A activity.

In both liver samples, the NO-Z and ND-Z formation rates were found to be monophasic on Eadie-Hofstee plots (Fig. 2), suggesting that a single enzyme mainly contributed to their respective generation. Furthermore, among the 22 liver samples, the generation of both...
metabolites was correlated, indicating that their metabolism may be performed by the same enzyme (Fig. 3).

**Screening of Zopiclone Metabolism with rH-CYP.** To identify the CYP isoform(s) involved in zopiclone metabolism, we incubated zopiclone (50 μM) with a panel of rH-CYP (Fig. 4). CYP2C8 was the isoform that displayed the highest enzyme activity for the formation rates of both zopiclone metabolites (Fig. 4A). However, when we calculated from these data the expected contribution of each CYP isoform in human liver microsomes the metabolism of zopiclone (Fig. 4B), we observed that CYP3A4 was the major enzyme involved in the NO-Z formation rate, followed by CYP2C9 and CYP2C8, whereas CYP2C8 remained the major CYP isoform involved in ND-Z, followed by CYP2C9 and CYP3A4.

Determination of zopiclone enzymatic kinetic constants could be obtained from rH-CYP2C8 (Fig. 5). Apparent $K_m$ and $V_{max}$ reached, respectively, 71 ± 6 μM and 2.5 ± 0.1 pmol/min/pmol CYP2C8 for ND-Z generation and 59 ± 9 μM and 1.0 ± 0.1 pmol/min/pmol CYP2C8 for NO-Z generation. When the intrinsic clearance of ND-Z (0.035 μl/min/pmol CYP2C8) was extrapolated to human liver, assuming that 1 mg of human liver microsome contains an average of 10 pmol of CYP2C8, it was found to represent ~60% of the intrinsic clearance obtained in the liver sample with medium CYP3A activity (0.35 versus 0.58 μl/min/mg). NO-Z intrinsic clearance (0.017 μl/min/pmol CYP2C8) obtained from recombinant CYP2C8, when extrapolated to human liver, represented 26% of the intrinsic clearance obtained in the liver sample with medium CYP3A activity (0.17 versus 0.64 μl/min/mg). Similar determinations could not be obtained from rH-CYP3A4 because of the low turnover number of this isoform toward the generation of both zopiclone metabolites.

**Effects of CYP Inhibitors on Zopiclone Metabolism in Human Liver Microsomes.** To clarify the contribution of the CYP isoforms outlined previously, we incubated zopiclone with three different human liver microsomes in the presence of different prototypic CYP inhibitors (Fig. 6). ND-Z generation was mainly inhibited by ketoconazole and sulfaphenazole, whereas NO-Z generation was only significantly inhibited by ketoconazole, suggesting the involvement of CYP3A and CYP2C in ND-Z formation and CYP3A in NO-Z formation (Table 1). Surprisingly, α-naphthoflavone, a CYP3A activator, did not increase the generation of zopiclone metabolites. CYP1A, CYP2D6, and CYP2E1 inhibitors had only minor effects on the extent of zopiclone metabolism.

**Correlation of Zopiclone Metabolite Generation to Different CYP Enzymatic Activities in Human Liver Microsomes.** To confirm the previous results, we correlated among the different human liver samples the generation of both zopiclone metabolites to classic CYP activities. We first observed for both metabolites a unique significant correlation with testosterone 6-β-hydroxylation (CYP3A activity) when zopiclone was incubated with the 22 liver samples at a final concentration of 200 μM ($r = 0.965$ and $r = 0.859$ for ND-Z and NO-Z, respectively; $p = .0001$; data not shown). Because zopiclone concentrations in humans never reach such high levels, we performed the same experiment at a more relevant concentration (25 μM; Table 2). The generation of both metabolites was always highly correlated to CYP3A4 activity, but the ND-Z formation rate was also correlated to CYP2C8 activity.
Discussion

In the present study, we report for the first time the in vitro metabolism of zopiclone in the presence of human liver microsomes and rH-CYP. We observed that CYP3A4 was the major enzyme involved in the generation of both zopiclone metabolites and that CYP2C8 was also involved in the ND-Z formation rate.

To obtain these results, we used the three in vitro main approaches, which can be used to determine the enzymes involved in the metabolism of a drug (Rodrigues, 1994): 1) selective inhibition of the main CYP enzymatic activities in human liver microsomes with specific CYP inhibitors; 2) correlation of the generation rates of the metabolites to classic predetermined CYP activities in different human liver samples; and 3) screening the metabolism of the drug with a panel of different heterologously expressed human CYP.

Zopiclone Metabolism in Human Liver Microsomes. We observed that zopiclone was metabolized into two major metabolites, ND-Z and NO-Z; this is consistent with the observation in humans (Goa and Heel, 1986). There was a very large interindividual variability in zopiclone metabolism from one liver sample to another, a characteristic that is in agreement with most of the drugs metabolized by CYP in humans and with the large variability of expression of most of the different human liver CYPs (Guengerich and Turvy, 1991; Shimada et al., 1994).

The metabolism of zopiclone into its two metabolites was found to be monophasic on Eadie-Hofstee plots, suggesting that its metabolism was probably mainly dependent on a single enzyme. CYP3A seemed to influence the generation of both zopiclone metabolites because the liver sample with the lowest CYP3A activity had a 10- and 3-fold
lower intrinsic clearance of ND-Z and NO-Z, respectively, compared with another liver sample with a medium CYP3A activity.

CYP inhibitors indicated that CYP3A was responsible for at least 40% of the metabolism of zopiclone into two metabolites and that the CYP2C subfamily accounted for about 40% of ND-Z formation. Correlation studies confirmed the major involvement of CYP3A4 in both zopiclone metabolites generation and the significant contribution of CYP2C8 in ND-Z formation.

**Zopiclone Metabolism in the Presence of rH-CYP**

Screening of zopiclone metabolism with a panel of different heterologously expressed human CYPs indicated that CYP2C8 had the highest enzymatic activity for the generation of both zopiclone metabolites. These results were adjusted to the relative content of the different CYP isoforms in human liver microsomes and indicated that: 1) CYP3A4 could be identified as the major isoform involved in NO-Z formation but not in ND-Z formation; 2) CYP2C8 was found to be the major CYP in ND-Z formation and had a lower contribution to NO-Z generation; and 3) CYP2C9 contributed significantly to the formation of both metabolites. However, our results cannot exclude the concomitant participation of other CYP isoforms, such as CYP2A6 and CYP2B6 or flavin monoxygenases, in zopiclone metabolism.

These results obtained with rH-CYPs are not in total agreement with those obtained with human liver microsomes; there was no involvement of CYP2C9 from the correlations studies, and CYP3A4 is the major isoform that metabolizes zopiclone into its two metabolites from inhibition and correlation studies. We have no clear explanation for such discrepancies, but we believe that the lack of validation of the different recombinant CYP isoforms may be one of the major hypotheses. Indeed, their enzymatic activity and affinity toward the substrate may be extremely different, depending on the ratio of recombinant CYP to recombinant cytochrome b5 and recombinant CYP reductase in each preparation (Rodrigues, 1994; Shet et al., 1995; Yamazaki et al., 1996a,b). Therefore, until complete validation of such recombinant devices by adapting their enzymatic activities to those found in human hepatic tissues, human liver microsomes should remain the gold standard for determining the enzymes involved in the metabolism of drugs. However, the intrinsic clearance of ND-Z determined with recombinant CYP2C8, representing 60% of the one obtained from the human liver sample with medium CYP3A activity, is in agreement with the results obtained with specific inhibitors; this concluded that CYP2C would represent ~40% of zopiclone metabolism.

Altogether, recombinant CYP studies confirmed the significant contribution of CYP3A4 and CYP2C8 in zopiclone metabolism.

**Zopiclone CYP-Dependent Drug Interactions in Humans**

Our in vitro results are in agreement with previous studies performed in humans that outlined the role of CYP3A in the metabolism of zopiclone. Itraconazole and erythromycin, two classical CYP3A inhibitors, were shown to significantly decrease the clearance of zopiclone (Aranko et al., 1994; Jalava et al., 1996). Rifampin, a classical CYP3A inducer, significantly increased zopiclone clearance (Villikka et al., 1997).

Thus far, CYP2C8 has never been involved in drug interactions concerning zopiclone. However, because we still do not know specific inhibitors of this isoform and because only very few drugs are presently identified as CYP2C8 substrates, such drug interactions may have been difficult to identify. Therefore, clinical relevance of the in vitro CYP2C8 involvement in zopiclone metabolism remains to be determined.

In conclusion, the present study characterizes for the first time the human CYP involved in vitro in zopiclone metabolism. Our results may help to prevent possible drug interactions associating zopiclone with other potent CYP3A inhibitors, such as ritonavir or clotrimazole (Quinn and Day, 1995; Bertz and Gunnemann, 1997).

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**References**


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