Characterization of Diuron N-Demethylation by Mammalian Hepatic Microsomes and cDNA-Expressed Human Cytochrome P450 Enzymes

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ABSTRACT:

Diuron, a widely used herbicide and antifouling biocide, has been shown to persist in the environment and contaminate drinking water. It has been characterized as a “known/likely” human carcinogen. Whereas its environmental transformation and toxicity have been extensively examined, its metabolic characteristics in mammalian livers have not been reported. This study was designed to investigate diuron biotransformation and disposition because metabolic routes, metabolizing enzymes, interactions, interspecies differences, and interindividual variability are important for risk assessment purposes. The only metabolic pathway detected by liquid chromatography/mass spectrometry in human liver homogenates and seven types of mammalian liver microsomes including human was demethylation at the terminal nitrogen atom. No other phase I or phase II metabolites were observed. The rank order of N-demethyldiuron formation in liver microsomes based on intrinsic clearance ($V_{\text{max}}/K_{m}$) was dog > monkey > rabbit > mouse > human > minipig > rat. All tested recombinant human cytochrome P450s (P450s) catalyzed diuron N-demethylation and the highest activities were possessed by CYP1A1, CYP1A2, CYP2C19, and CYP2D6. Relative contributions of human CYP1A2, CYP2C19, and CYP3A4 to hepatic diuron N-demethylation, based on average abundances of P450 enzymes in human liver microsomes, were approximately 60, 14, and 13%, respectively. Diuron inhibited relatively potently only CYP1A1/2 ($IC_{50}$ 4 $\mu$M). With human-derived and quantitative chemical-specific data, the uncertainty factors for animal to human differences and for human variability in toxicokinetics were within the range of the toxicokinetics default uncertainty/safety factors for chemical risk assessment.

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is a substituted phenylurea compound widely used as a systemic herbicide and antifouling biocide. Because of its extremely slow breakdown in water, diuron is highly persistent in the environment (Madhum and Freed, 1987). Its wide use in agriculture leads to pollution of the freshwaters by soil leaching, and it has been detected in marinas and coastal areas in the United Kingdom (Thomas et al., 2001), Germany (Lamoree et al., 2002), and Japan (Okamura et al., 2003). The European Water Framework Directive includes diuron as a priority substance that represents a significant risk to or through the European aquatic environment (European Commission, 2001). The U.S. Environmental Protection Agency (2005) also reported diuron on the list of substances considered contaminants of drinking water since 1998. Diuron has been classified as a slightly hazardous (toxicity class III) pesticide by World Health Organization. The acute oral LD$_{50}$ for male rats is 4721 mg/kg. Diuron has been characterized as a “known/likely” human carcinogen on the basis of urinary bladder carcinomas in both sexes of the Wister rat, kidney carcinomas in the male rat (a rare tumor), and mammary gland carcinomas in the female NMRI mouse (U.S. Environmental Protection Agency, 2004). Moreover, diuron also showed in vivo mutagenic activity in Swiss mice by the bone marrow micronucleus test (Agrawal et al., 1996).

The environmental impact of diuron transformations was recently reviewed by Giacomazzi and Cochet (2004). Among the detected transformation products, at least dichloroaniline, the principal product of biodegradation, exhibited a higher toxicity and persistence in soil, water, and groundwater. Demethyldiuron had higher nontarget toxicity than diuron (Tixier et al., 2000).

Only a few studies on diuron metabolism in mammals have been published. In metabolic studies of rats and dogs, N-(3,4-dichlorophenyl)urea was the predominant metabolite in the urine. Small amounts of N-(3,4-dichlorophenyl)-N-methylurea, 3,4-dichloroaniline, 3,4-dichlorophenol, and unchanged diuron were detected (Hodge et al., 1967).

In a human postmortem case diuron and its demethylated, didem-
ethylated, and hydroxylated metabolites were identified in plasma and urine. Diuron levels as high as 5 mg/liter in plasma and 3 mg/liter in urine have been found; moreover, a rough estimate of the total concentration of diuron plus metabolites in plasma was near 100 mg/liter, resulting in an estimated uptake of at least several grams (Verheij et al., 1989). On the other hand, in a hospitalized patient, diuron was completely metabolized, mainly via demethylation (2.0 and 180 mg/liter) and didemethylation (70 and 68 mg/liter) in blood and urine, respectively. In urine, ethanol extracts contained 36 mg/liter hydroxyphenyldiuron and 0.2 mg/liter 3,4-dichloroaniline (Van Boven et al., 1990).

The cytochrome P450 (P450) enzymes are important in the metabolism of various endogenous substrates as well as a wide range of xenobiotics. During the last few years several articles on the role of P450s in the metabolism of a wide variety of pesticides have appeared (Usmani et al., 2004; Much and Williams, 2006; Abass et al., 2007a,b). On the other hand, only limited data are available on diuron biotransformation by mammalian P450s. Suzuki and Casida (1981) reported that mouse liver microsomal oxidase converted diuron into seven metabolites. The major metabolite was N-demethylated, and minor amounts of three hydroxymethyl metabolites and two formamides were detected by thin-layer chromatography.

Studies on diuron biotransformations in different mammalian species including humans are vital for chemical risk assessment purposes by the application of the toxicokinetics default uncertainty/safety factors for interspecies differences and interindividual variation (Renwick and Lazarus, 1998). However, there are no data on the mammalian hepatic enzyme kinetics of diuron, neither on the contribution of P450 isoforms to its metabolic pathways nor on the inhibitory interaction with different human liver P450 enzymes. The current study was conducted 1) to identify and quantify potential diuron metabolites by human liver homogenates and human, mouse, rat, dog, monkey, minipig, and rabbit liver microsomes in vitro, 2) to assess the relative contributions of human P450s responsible for diuron metabolism, 3) to quantify diuron metabolites by individual human liver microsomes, and 4) to examine the inhibitory interactions with different human liver P450 enzymes.

Materials and Methods

**Chemicals.** Diuron, N,N’-bis(3,4-dichlorophenyl)urea, and 3-(3,4-dichlorophenyl)-1-methylurea were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and 3,4-dichloroaniline, bis(p-chlorophenyl)urea, and 3-(4-chlorophenyl) methyl urea were from ChemService (West Chester, PA). Midazolam was a kind gift from F. Hoffman-La Roche (Basel, Switzerland), and omeprazole was a gift from Astra Zeneca (Mölndal, Sweden). HPLC-grade solvents were obtained from Rathburn (Walkerburn, UK) and Labscan (Dublin, Ireland). All other chemicals used were from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. Water was freshly prepared in-house with the Simplicity 185 (Millipore S.A., Molsheim, France) water purification system and was UP grade (ultra pure, 18.2 MΩ).

**Human Liver Homogenates, Mammalian Liver Microsomes, and cDNA-Expressed Human P450 Enzymes.** Human liver samples used in this study were obtained from the University Hospital of Oulu as surplus from kidney transplantation donors. The collection of surplus tissue was approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Finland. All liver samples were from individuals of Caucasian race including four females and six males between the ages of 21 and 62 years. Intracerebral hemorrhage was the primary cause of death. Detailed characteristics of the liver samples are presented in our previous publication (Abass et al., 2007a). The livers were transferred to ice immediately after the surgical excision and cut into pieces, snap-frozen in liquid nitrogen, and stored at −80°C. Human liver homogenate was prepared from livers of 10 individuals by homogenizing liver tissue in 4 volumes of 0.1 M phosphate buffer (pH 7.4); i.e., the homogenate contained 200 mg of hepatic tissue/ml. Male DBA/2 mouse, Sprague-Dawley rat, Beagle dog, cynomolgus monkey, Göttingen minipig, and New Zealand White rabbit liver samples were obtained after approval of the Ethics Committee of the University of Oulu, Finland. All microsomes were prepared by standard differential ultracentrifugation (Pekkonen et al., 1974). The final microsomal pellet was suspended in 100 mM phosphate buffer, pH 7.4. Protein content was determined by the Bradford method (Bradford, 1976). Baculovirus insect cell-expressed human P450s (CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) were purchased from BD Biosciences Discovery Labware (Bedford, MA).

**In Vitro Assay of Diuron Metabolites.** The standard incubation mixture contained serial concentrations of diuron (final concentrations of 5, 25, and 100 μM), 0.1 mg of pooled liver microsomal protein, and 1 mM NADPH in a final volume of 200 μl of 0.1 M phosphate buffer (pH 7.4). Diuron was first dissolved in dimethyl sulfoxide (DMSO) (final amount in the reaction medium 1%). After a 2-min incubation at +37°C in a shaking incubator block (Thermomixer 5436, Eppendorf AG, Hamburg, Germany) the reaction was started by adding NADPH. The mixture was incubated at +37°C for 20, 40, and 60 min, and the reaction was stopped with 200 μl of ice-cold acetonitrile containing an internal standard. All incubations were performed in triplicate. After centrifugation at 10,000g for 15 min, the supernatant was collected and stored at −20°C until analyzed.

To measure the production of potential metabolites, human liver homogenates containing the same final diuron concentrations as the microsomal incubations were prepared. In addition to 40 μM of human liver homogenates (contains approximately 0.14 mg of microsomal protein), the other components in homogenate incubates were 5 mM uridine 5’-diphosphoglucuronic acid, 1 mM glutathione, 1.2 mM adenosine-3’-phosphate-5’-phosphosulfate, and 1 mM NADPH in a final volume of 200 μl of 0.1 M phosphate buffer (pH 7.4). The incubations and analytical method were similar to those for the microsomal incubations.

To measure the main diuron metabolites of recombinantly expressed P450 enzymes, the standard incubation mixture (200 μl) contained 0.1 M phosphate buffer (pH 7.4), 1 mM NADPH, 100 μM diuron, and recombinantly expressed P450 enzymes (50 pmol P450/ml). Incubations were carried out according to the manufacturer’s instructions. In brief, the reaction was started by adding recombinant enzymes to the preincubated reaction mixture (2 min at +37°C), mixed gently, and incubated for 30 min at +37°C in an incubator block without agitation. Otherwise, the incubation protocol and analytical method were similar to those for microsomal incubations.

**Kinetic Parameters.** To measure the enzyme kinetic parameters in both the microsomal samples and recombinantly expressed P450 enzymes, the standard incubation mixture contained diuron (final concentrations 1.0–400 μM). Incubation mixtures and methods were the same as those mentioned above, except that the incubation times were 20 min for microsomal samples and 30 min for rP450s. Samples were analyzed by LC/MS-MS. The kinetic parameters \( V_{\text{max}} \) and \( K_{\text{m}} \) were calculated using Prism 5.0 (GraphPad Software, Inc., San Diego, CA) by nonlinear regression. These values were used to calculate the intrinsic clearance value (\( V_{\text{max}}/K_{\text{m}} \)). All results are expressed as means ± S.E. for three replicates. In the standard experimental conditions used for diuron demethylation, the reaction rate of diuron demethylation was linear at least up to 0.1 mg of microsomal protein/ml and 60 min incubation time.

**Chromatography of Diuron Metabolites.** Before analysis, samples were centrifuged for 10 min at 13,400g with an Eppendorf MiniSpin centrifuge (Eppendorf AG). Chromatographic separation was carried out with the Waters Alliance 2690 HPLC system (Waters, Milford, MA). The column used was a Waters X Terra MS C18 (2.1 mm × 50 mm, particle size of 3.5 μm) together with a Phenomenex C18 2.0 mm × 4.0 mm precolumn (Phenomenex, Torrance, CA). The temperature of the column oven was 30°C. The eluent flow rate was 0.3 ml/min. The eluents used were ultrapure-grade water containing 0.1% acetic acid (A) and acetonitrile (B). A linear gradient elution from 10% B to 84% B in 7 min was applied. Solvent B was thus maintained at 95% for 1 min before reequilibration (6 min). The total analysis time was 14 min.

**Mass Spectrometry.** The initial screening of the compounds present and accurate mass measurements were carried out using a Micromass LCT (Micromass, Altrincham, UK) time of flight mass spectrometer equipped with a Z-spray ionization source. A generic positive electrospray ionization method was used for all substrates and metabolites. The capillary voltage was 3500 V, cone voltage was 25 V, and desolvation and source temperatures were 250 and
150°C, respectively. Nitrogen was used as the desolvation and cone gas with flow rates of 750 and 150 liters/h. The mass spectrometer and HPLC system were operated under Micromass MassLynx 3.4 software. For exact mass measurements the lock mass was leucine enkephalin ([M + H]$^+$ at m/z 556.2771), and it was delivered into the ionization source through a T-junction using a syringe pump (Harvard Apparatus Inc., Holliston, MA).

The quantification (multiple reaction monitoring) and fragmentation measurements were performed with a Micromass Quattro II triple quadrupole instrument equipped with a Z-spray ionization source. The capillary voltage was 3500 V, cone voltage was 25 V, and desolvation and source temperatures were 250 and 150°C, respectively. Collision energies were 15 eV for 3-(3,4-dichlorophenyl)-1-methylurea (N-demethyl diuron) and 25 eV for diuron. The internal standard was 3-(4-chlorophenyl)methylurea, and its collision energy was 15 eV. The collision gas was argon with collision-induced dissociation gas cell pressure 1.7 × 10\(^{-3}\) mbar. Nitrogen was used as the drying and nebulizing gas with flow rates of 450 and 15 liters/h. The fragmentation reactions monitored (multiple reaction monitoring) were from m/z 185 to m/z 128 for the internal standard, from m/z 219 to m/z 162 for N-demethyl diuron and from m/z 233 to m/z 160 for diuron. External standards were measured in the beginning, middle, and end of the experiment to ensure the quality of the analysis. The lower limit of quantitation was 0.1 μM for all compounds. Intraday coefficients of variation were less than 15% throughout the quantitation range of 0.1 to 50 μM.

**Inhibition Assays.** Metabolites of bupropion, amodiaquine, tolbutamide, omeprazole, dextromethorphan, chlorozoxazone, and midazolam were analyzed by a Shimadzu VP series high-performance liquid chromatograph with an autoinjector (Shimadzu, Kyoto, Japan). The analytical column was a Waters Symmetry C\(_{18}\) (3.9 mm × 150 mm, particle size of 5 μm) together with a Lichospher 100 RP-18 4.0 mm × 4.0 mm precolumn (Merck, Darmstadt, Germany). Chromatographic methods were isocratic, except in the case of omeprazole when a linear gradient elution from 15 to 35% A in 8 min was used. Mobile phases were pumped at a flow rate of 1.0 ml/min. The injection volume used was 20 μl. The concentrations of metabolites were calculated from peak height ratios of the UV chromatograms on the basis of standard calibration curves of authentic metabolites. Metabolites of ethoxyresorufin, ethoxycoumarin, coumarin, and pentoxyresorufin were analyzed fluorometrically. All of the incubation and analysis conditions are summarized in Table 1.

Diyuron was added in different concentrations (final concentrations in the incubation mixture were 5–100 μM) to the incubation mixture with a small volume of DMSO as solvent. Fresh DMSO dilutions from stock solution of DMSO were used for each assay. The final amount of DMSO was 1% in incubation mixtures. For chlorozoxazone 6-hydroxylation the solvent was evaporated because of the potent inhibition effect of DMSO on CYP2E1 (Pelkonen et al., 1998), and the residue was diluted with 10 μl of lipid solution. Lipid solution was prepared by adding 8 μl of dimethyldioctadecylammoniumbromide in chloroform (100 mg/ml) to 200 μl 1-α-phosphatidylethanolamine dioleryl (C18:1(Cis)-9) in chloroform. This mixture was evaporated with a stream of nitrogen flow, 2 ml of water was added, and the final mixture was sonicated (Branson Sonifier 250 GWB) on an ice bath for 10 min by using 50% power.

The enzyme activities in the presence of diyuron were compared with the control incubations into which only solvent was added. The IC\(_{50}\) values for inhibitors (concentration causing 50% reduction of control activity) were determined from duplicate incubations by linear regression analysis from the plot of the logarithm of inhibitor concentration versus percentage of the activity remaining after inhibition using Origin 6.0 (OriginLab Corp., Northampton, MA).

**Correlation with Model P450 Substrate Activities.** A bank of 10 livers was used to assess the metabolism of diuron in individual livers as well as to correlate the activities with model P450 substrate activities. A correlation was performed between the formation of N-demethyl diuron and each P450 activity across the human liver bank. Model substrate reactions used for correlations were the same as those used in the inhibition studies above. For all data points the means of duplicate incubations were used. Bivariate linear Pearson’s correlation coefficients (r) were calculated between metabolite formations and model activities in livers.
Results

Identification of Diuron Metabolites Produced in Vitro by Human Liver Homogenates and Mammalian Liver Microsomes. Incubations of human liver homogenates (HLH) and human (HLM), rat (RLM), mouse (MLM), dog (DLM), monkey (MonLM), minipig (PLM), and rabbit (RabLM) liver microsomes with various concentrations of diuron were analyzed by LC/time-of-flight/MS.

Only N-demethyldiuron was identified from the extracted mass chromatograms. No other potential metabolite was observed, even with 3,4-dichloroaniline, 3-(4-chlorophenyl)methylurea, 3,4-dichlorophenylurea, bis(p-chlorophenyl)urea, and N,N'-bis(3,4-dichlorophenyl)urea as reference standards. The exact masses and biotransformation by cytochrome P450-mediated diuron N-demethylation in the context of the overall scheme of human in vivo metabolism are presented in Fig. 1.

Fragmentations of the metabolite were determined by triple quadrupole MS and the collision energy used was 25 eV. For N-demethyldiuron (M+ = 219) the main fragments were at m/z 162 and 127, whereas fragments for diuron (M+ = 233) were at m/z 188 and 160.

Metabolism of Diuron in Vitro by Human Liver Homogenates and Mammalian Liver Microsomes. Diuron and N-demethyldiuron concentrations as a function of incubation time (20, 40, and 60 min) using HLM, RLM, MLM, DLM, MonLM, PLM, RabLM, and HLH and at diuron concentrations of 5, 25, and 100 μM were analyzed by LC/MS-MS. The results are presented in Fig. 2.

A concentration of 100 μM diuron incubated for 20, 40, and 60 min) with DLM produced the highest concentrations of N-demethyldiuron (27.4, 37.8, and 43.2 μM, respectively), whereas PLM produced the lowest (3.3, 5.7, and 7.0 μM, respectively). The corresponding concentrations of N-demethyldiuron were 5.8, 8.9, and 10.8 μM in HLM and 14.5, 19.0, and 22.1 μM in HLH. This difference in human liver preparations is due to the fact that the homogenates added to the incubations contained a slightly higher amount of microsomal protein (final microsomal protein concentration 0.14 mg/ml) than when microsomal preparations were added to the incubation mixtures (final protein concentration 0.1 mg/ml). No phase II enzyme-associated metabolites were observed.

Alongside N-demethyldiuron formation, the reduction in the amount of the parent compound was measured. It is worthy to note that at the lowest diuron concentration, very little further metabolism of N-demethyldiuron was observed with RabLM, in contrast to HLH and DLM, in which very fast disappearances of both metabolite and parent compound were detected. Moreover, DLM had the same disappearance trend when 25 μM diuron was incubated, whereas the N-demethyldiuron was increased, roughly corresponding to diuron disappearance in the other species. At the highest diuron concentration, the N-demethyldiuron concentrations were increased by time in
all mammalian liver sample preparations in correspondence to disappearances of the parent compound, and DLM was the most active in diuron transformation over time.

Kinetic Parameters of the Diuron Metabolism in Mammalian Liver Microsomes. N-Demethyldiuron was quantified by use of a triple quadrupole mass spectrometer as a result of diuron metabolism by all mammalian liver microsomes, whereas RLM had the lowest (75 μl/mg of protein/min). The HLM intrinsic clearance value was 174 μl/mg of protein/min. The rank order of the intrinsic clearance values in different species was DLM > MonLM > RabLM > MLM > PLM > HLM > RLM.

Identification of the Human P450s Responsible for Diuron Metabolism. A screen of 11 human recombinant P450s (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) showed that all tested P450s had detectable diuron N-demethylation activities. The kinetic parameters for the rP450s were determined and are shown in Table 3.

CYP2C19 showed the highest affinity toward diuron demethylation, corresponding to the lowest $K_m$ value, whereas the lowest affinity was observed for CYP2C8. $V_{\text{max}}$ values for CYP1A1 and CYP1A2 were the highest (215.6 and 173.4 nmol of N-demethyldiuron/mg of protein/min), whereas the $V_{\text{max}}$ of CYP2C8 was the lowest (1.07 nmol of N-demethyldiuron/nmol P450/min). $V_{\text{max}}/K_m$ values illustrated that CYP1A1, CYP1A2, and CYP2C19 were the most efficient rP450s for diuron transformation to N-demethyldiuron (2395.6, 1968.2, and 1696.7 μl/nmol P450/min, respectively), whereas CYP2C8 was the least efficient (6.2 μl/nmol P450/min).

Taking into account the average human hepatic microsomal protein amounts of P450s (CYP1A2, 52; CYP2A6, 36; CYP2B6, 11; CYP2C8, 24; CYP2C9, 73; CYP2C19, 14; CYP2D6, 8; CYP2E1, 61; and CYP3A4, 111 pmol/mg microsomal protein) (Rostami-Hodjegan et al., 2007) and the actual intrinsic clearance values for various P450s, the relative contributions of each P450 enzyme were calculated and are shown in Table 3.

Quantification of Diuron Metabolism in Vitro by Individual Human Liver Microsomes. Assays were performed with 25 μM diuron and a 20 min incubation time, and metabolite formation was measured in 10 individual HLM. N-Demethyldiuron formation rates varied from 0.08 to 1.57, i.e., approximately a 20-fold variation, and the mean value was 1.05 nmol of N-demethyldiuron/mg of protein/min (Table 4). The specific activities of P450 isoform-catalyzed reactions in microsomes from 10 human livers and their correlations with N-demethyldiuron formation were studied. The highest correlations between N-demethyldiuron formation and CYP activities were seen with CYP1A2 and CYP2C8, with correlation coefficients ($r = 0.35$). With all other P450s the correlations were <0.35.

Inhibitory Interactions with Different Human Liver P450s. The effects of diuron on P450-selective activities were determined in human liver microsomes. The only significant IC$_{50}$ values were 4.0 μM for 7-ethoxyresorufin-O-deethylation (multiple P450s) and 4.1 μM for 7-ethoxycoumarin-O-deethylation (CYP1A1/2). All other values for CYP2A6, CYP2B, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were higher than 100 μM, indicating very low or absent affinity.

Discussion

Diuron has been reported to be metabolized in vivo in rats, dogs (Hodge et al., 1967), and humans (Verheij et al., 1989; Van Boven et al., 1990) via demethylation, didemethylation, and hydroxylation. In addition, mouse liver microsomes metabolized diuron mainly to demethyldiuron (Suzuki and Casida, 1981). In the present study, the only diuron biotransformation pathway observed in the seven types of
mammalian hepatic microsomes and human liver homogenates was demethylation at the terminal nitrogen atom, even if screening of the potential metabolites had been performed by LC/MS. It is possible that other potential metabolic pathways reside in extrahepatic tissues or need much longer incubation times or different incubations conditions than those we used with hepatic preparations. Also, diuron doses in human poisoning cases, in which other human metabolites were observed, were very high.

*N*-Demethyldiuron formation proceeds via the oxidation of *N*-methyl to the *N*-hydroxymethyl [\(-\text{N}(\text{CH}_3)\text{CH}_2\text{OH}\)], followed by further oxidation to the formamide [\(-\text{N}(\text{CH}_3)\text{CHO}\)]. The *N*-hydroxy derivative was reported to be very unstable, and it undergoes partial conversion to *N*-demethyldiuron (Suzuki and Casida, 1981). In a human postmortem case, the hydroxymethyl metabolite was detected from a chemical ionization spectrum using LC/MS with a moving-belt interface, even though it was poorly separated from the didemethylated metabolite, and its amount was small. However, in our study, the hydroxymethyl metabolite was not detected in any species even though they were searched from extracted mass chromatograms. If concentration may be too small to detect or it may be too unstable even for electrospray ionization.

*N*-Demethyldiuron was detected and quantified in all tested liver preparations from different mammalian species. Because the concen-
trations of diuron and its $N$-demethylation metabolite were decreasing, especially at the lowest diuron concentration, further metabolism could have happened in MLM, DLM, MonLM, and HLH, although we did not detect any metabolites. In electrospray ionization, all of the compound would not necessarily ionize, and, consequently, these metabolites would not be detected. In some dogs diuron was reported to be metabolized in vivo to phenolic compounds that are frequently excreted as sulfate and glucuronide conjugates (Hodge et al., 1967). Even if we were not able to detect any phase II metabolites, it is possible that the formation of “MS-invisible” metabolites could partially explain the rapid disappearances of both diuron and $N$-demethyl-diuron, at least in dogs.

An animal to human efficiency comparison revealed that DLM metabolized diuron 2.3-fold more than HLM, whereas the RLM had the smallest value (0.43-fold). Differences in efficiencies between rat, minipig, and human liver microsomes have been reported by Lang et al. (1996) in the metabolism of triazine herbicides such as atrazine and ametryn. Also, with other pesticides such as chlorpyrifos (organophosphorus insecticide) and carbofuran (carbamate insecticide), human liver microsomes showed lower rates of metabolism than mouse and rat liver microsomes (Tang et al., 2001; Usmani et al., 2004).

The interspecies differences in $N$-demethyl-diuron formation as a function of time correlated with the variation in kinetic parameters. The most active mammalian microsomes in diuron biotransformation over the time and concentrations used were DLM. However, in the 5 and 25 μM diuron incubations, $N$-demethyl-diuron concentrations were lower because of a massive disappearance of the parent compound. Also kinetic data demonstrated that DLM had the highest capacity and efficiency for diuron transformation. On the other hand, RLM were the least active microsomes in diuron transformation over time and had the lowest efficiency. HLM displayed the highest affinity and lowest capacity.

All tested recombinant P450s were able to metabolize diuron to $N$-demethyl-diuron. Kinetic characterization showed that diuron metabolism to $N$-demethyl-diuron was one-phasic; in other words, it can be described as involving one active site or several sites with similar enzyme kinetic characteristics. CYP1A1, CYP1A2, CYP2C19, CYP2D6, CYP2B6, and CYP3A4 were relatively active in $N$-demethyl-diuron formation. On the basis of intrinsic clearance values and human hepatic microsomal P450 isoenzyme concentrations, the relative contribution of CYP1A2 to diuron $N$-demethylation was estimated to be 60% because of both high intrinsic clearance and average amount. CYP2C19 was estimated to contribute 14% and CYP3A4 13%. Although CYP1A1 had the highest intrinsic clearance value, its contribution is probably negligible because the amount of CYP1A1 in the human liver is very small (Rendic and Di Carlo, 1997). However, the contribution of CYP1A1 in extrahepatic tissues may be considerable in special circumstances. The rank order of the relative contributions of hepatic P450 enzymes was CYP1A2 > CYP2C19 > CYP3A4 > CYP2D6 > CYP2B6 > CYP2C9 > CYP2E1 > CYP2A6 > CYP2C8.

It is an established fact that xenobiotic metabolizing P450 enzyme activities display a large interindividual variation (Pelkonen et al., 1998). Also in this study, specific activities of P450 isof orm-catalyzed reactions in microsomes from 10 human livers displayed high variability. An individual with the highest levels of both CYP1A2 and CYP2C19 (HL28) had the highest $N$-demethyl-diuron formation, and the reverse was true for an individual (HL31) who had the lowest levels of both isof orms. Moreover, the correlation studies in individual humans suggested that at least those individuals with a high CYP1A2 content may be able to metabolize diuron more efficiently. A high correlation with CYP2C8 activity was probably an artifact, because CYP2C8 is very poor in $N$-demethylating diuron.

Extensive in vitro screening of potential P450-based interactions indicated that diuron inhibited CYP1A1/2 enzymes in HLM relatively potently, with an IC₅₀ value of 4.1 μM. The corresponding values were higher than 100 μM for the other tested P450s. It is not surprising that diuron inhibited CYP1A1/2, as these were P450 isoforms with a relatively low Kₘ value for diuron $N$-demethylation. Very little has been reported about the potential interaction of diuron with other P450 enzyme activities. Diuron did not affect the CYP1A2-associated aromatase activity in human placental microsomes in vitro (Vinggaard et al., 2000).

The 100-fold uncertainty factor (UF) is used to convert a no-observed-adverse-effect level from an animal toxicity study to a safe value for human intake. Furthermore, this UF has to allow for a 10-fold interspecies difference [which is subdivided into a factor of 10⁰·⁷ (4.0) for toxicokinetics and 10⁻⁰·⁴ (2.5) for toxicodynamics] and a 10-fold interindividual variation [which is divided equally into two subfactors each of 10⁻⁰·⁴ (3.16)] (World Health Organization/International Programme on Chemical Safety, 2005). In our results the highest uncertainty factor for animal to human differences in toxicokinetics (AKUF) was 2.3-fold. In addition, the uncertainty factor for human variability in toxicokinetics (HKUF) was 1.5-fold, as defined by Renwick and Lazarus (1998) as a variation between the mean and the highest value. It has to be stressed here that we measured only the hepatic metabolism of diuron, but, on the other hand, metabolism is usually the most important factor contributing to interindividual and interspecies differences in toxicokinetics.

To obtain quantitative toxicokinetic data for comparison between individuals or between animals and human, human data are needed (Falk-Filipsson et al., 2007). Moreover, risk assessment has to be carried out using quantitative chemical-specific data that will influence the toxicokinetics and toxicodynamics (Walton et al., 2001). Our studies, although restricted to metabolic data by human and animal liver preparations, provide important quantitative diuron specific data for risk assessment, which suggest that both the interspecies difference and interindividual variation values are within the standard toxicokinetic default uncertainty/safety factors. These results will be valuable in further defining the risks associated with exposure to diuron.

In conclusion, metabolic route, kinetics, and interactions studies showed that the diuron biotransformation pathway involved demethylation at the terminal nitrogen atom in seven different mammalian hepatic microsomes and in human liver homogenates. No phase II metabolites were observed. All tested rP450s catalyzed diuron $N$-demethylation. The most important human liver P450s were CYP1A2, CYP2C19, and CYP3A4. Diuron was a relatively potent inhibitor of human CYP1A1/2, which might be of significance at least in occupational situations in which workers are exposed to higher diuron concentrations. The interspecies differences and interindividual variation were within the range of usual toxicokinetics default uncertainty/safety factors for chemical risk assessment. Our quantitative data on interspecies differences and individual variability may have an important contribution to diuron risk assessment.

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