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CHARACTERIZATION OF DIURON N-DEMETHYLATION BY MAMMALIAN HEPATIC MICROSOMES AND *c*DNA-EXPRESSED HUMAN CYTOCHROME P450 ENZYMES.

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Abbreviations: CYP, cytochrome P450; HLM, pooled human liver microsomes; RLM, rat liver microsomes; MLM, mouse liver microsomes; DLM, dog liver microsomes; MonLM, monkey liver microsomes; PLM, minipig liver microsomes; RabLM, rabbit liver microsomes; HLH, human liver homogenates; UF, uncertainty factor; AK_{UF}, uncertainty factor for animal to human differences in toxicokinetics; AK_{UF}, uncertainty factor for human variability in toxicokinetics; HPLC, high-performance liquid chromatograph.

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ABSTRACT

Diuron, a widely used herbicide and antifouling biocide, has been shown to persist in the environment and contaminate the drinking water. It has been characterized as a "known/likely" human carcinogen. While the environmental transformation and toxicity have been extensively examined, metabolic characteristics in mammalian livers have not been published. 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 LC–MS in human liver homogenates and seven 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_{max}/K_m) was dog > monkey > rabbit > mouse > human > minipig > rat. All tested recombinant human CYPs 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 CYP enzymes in human liver microsomes, were about 60, 14 and 13%, respectively. Diuron inhibited relatively potently only CYP1A1/2 (IC_{50} value 4 μM). Using the human-derived and quantitative chemical-specific data, the uncertainty factor for animal to human differences (AK_{UF}) and for human variability (HK_{UF}) in toxicokinetics were within range of the toxicokinetics default uncertainty/safety factors for chemical risk assessment.

INTRODUCTION

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is a substituted phenylurea compound widely used as a systemic herbicide and antifouling biocide. Due to 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 (WFD) includes diuron as a priority substance (PS) which represents a significant risk to or through the European aquatic environment (European Commission, 2001). The United States Environmental Protection Agency also reported diuron on the list of substances considered contaminants of drinking water since 1998 (USEPA, 2005).

Diuron has been classified as a slightly hazardous (toxicity class III) pesticide by WHO. The acute oral LD_{50} for male rats is 4721 mg/kg. Diuron has been characterized as a "known/likely" human carcinogen based on 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 (USEPA, 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 non-target 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, didemethylated and hydroxylated metabolites were identified in plasma and urine. Diuron levels as high as 5 mg/L in plasma and 3 mg/L in urine have been found, moreover, a rough estimate of the total concentration of diuron plus metabolites in plasma was found to be near 100 mg/L, resulting in as estimated uptake of at least several grams (Verheij et al., 1989). On the other hand, in a hospitalized case, diuron was completely metabolized mainly via demethylation (2.0 and 180 mg/L) and didemethylation (70 and 68 mg/L) in blood and urine, respectively. In addition urine extracts contained 36 mg/L hydroxyphenyldiuron and 0.2 mg/L 3,4-dichloroaniline (Van Boven et al., 1990).

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

The studies on the diuron biotransformations in different mammalian species including human 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 is no data concerning the mammalian hepatic enzyme kinetics of diuron, neither the contribution of CYP isoforms to its metabolic pathways

nor the inhibitory interaction with different human liver CYP 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 CYPs 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 CYP enzymes.

MATERIALS AND METHODS

Chemicals. Diuron [3-(3,4-dichlorophenyl)-1,1-dimethyl urea], N,N'-bis(3,4-dichlorophenyl)urea and 3-(3,4-dichlorophenyl)-1-methylurea were purchased from Dr. Ehrenstorfer (Augsburg, Germany), and 3,4-dichloroaniline, bis(p-chlorophenyl)urea and 3-(4-chlorophenyl) methyl urea from ChemService (West Chester, PA). Midazolam was a kind gift from F. Hoffmann La Roche (Basel, Switzerland) and omeprazole from Astra Zeneca (Mölndal, Sweden). HPLC-grade solvents were obtained from Rathburn (Walkerburn, UK) and Labscan (Dublin, Ireland). All other chemicals used were from the Sigma Chemical Company (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 M Ω).

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 of Caucasian race including 4 female and 6 male between age of 21 and 62. 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 ten individuals by homogenizing liver tissue in four 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 (Pelkonen 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 CYPs (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 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 dimethylsulfoxide (DMSO; final amount in the reaction medium 1.0 %). After a 2 min incubation at +37 °C in a shaking incubator block (Eppendorf Thermomixer 5436, Hamburg, Germany) the reaction was started by adding NADPH. The mixture was incubated at +37 °C for 20, 40 and 60 minutes and the reaction was stopped with 200 µl of ice cold acetonitrile containing an internal standard. All incubations were carried out in triplicate. After centrifugation at 10000 × *g* for 15 min the supernatant was collected and stored at -20 °C until analyzed.

To measure the production of potential metabolites, human liver homogenates incubations were prepared containing the same final diuron concentrations as the microsomal incubations. In addition to 40 µL of human liver homogenates (contains app. 0.14 mg microsomal protein), the other components in homogenate incubates were 5 mM uridine 5`-diphosphoglucuronic acid (UDPGA), 1 mM glutathione, 1.2 mM adenosine-3`-phosphate-5`-phosphosulfate (PAPS) 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 the microsomal incubations.

To measure the main diuron metabolites of recombinantly expressed CYP 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 CYP enzymes (50 pmol CYP *per* ml). Incubations were carried out according to the manufacturer's instructions. Shortly, 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 microsomal incubations.

Kinetic parameters. To measure the enzyme kinetic parameters in both the microsomal samples and recombinantly expressed CYP enzymes, the standard incubation mixture contained diuron (final concentrations 1.0 – 400 µM). Incubation mixtures and methods were the same as mentioned above, except the incubation times were 20 min for microsomal samples and 30 min for rCYPs. Samples were analyzed by LC-MS-MS. The kinetic parameters V_{max} and K_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_{max}/K_m). All results are expressed as mean ± standard error for three replicates. In the standard experimental conditions used for diuron demethylation, reaction rate of diuron demethylation was linear at least up to 0.1 mg of microsomal protein/ml and 60 minute incubation time.

Chromatography of the diuron metabolites. Before analysis, samples were centrifuged for 10 minutes at 13400 × g with an Eppendorf MiniSpin centrifuge (Eppendorf AG, Hamburg, Germany). Chromatographic separation was carried out with the Waters Alliance 2690 HPLC system (Waters Corp., Milford, MA). The column used was a Waters XTerra MS C18 (2.1 mm x 50 mm, particle size of 3.5 µm) together with a Phenomenex C18 2.0 mm x 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

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% B to 84 % B in 7 min was applied. Solvent B was thus maintained at 95 % for 1 min before re-equilibration (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 (TOF) 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 25 V, and desolvation and source temperatures 250 and 150°C, respectively. Nitrogen was used as the desolvation and cone gas with flow rates of 750 and 150 L/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-union using a syringe pump (Harvard Apparatus, Holliston, MA).

The quantification (multiple reaction monitoring, MRM) 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 25 V, and desolvation and source temperatures 250 and 150°C, respectively. Collision energies were 15 eV for 3-(3,4-dichlorophenyl)-1-methylurea (N-demethyldiuron) 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 CID gas cell pressure $1{,}7 \times 10^3$ mbar. Nitrogen was used as the drying and nebulizing gas with flow rates of 450 and 15 L/h. The fragmentation reactions monitored (MRM) were from m/z 185 to m/z 128 for the internal standard, from m/z 219 to m/z 162 for N-demethyldiuron 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.

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Intraday coefficients of variation were less than 15% throughout the quantitation range of 0.1 –50 μM.

Inhibition assays. Metabolites of bupropion, amodiaquine, tolbutamide, omeprazole, dextromethorphan, chlorzoxazone and midazolam were analyzed by a Shimadzu *VP* series HPLC with an auto injector (Shimadzu, Kyoto, Japan). The analytical column was a Waters Symmetry C_{18} (3.9 mm x 150 mm, particle size of 5 μm) together with a Lichospher 100 RP-18 4,0 mm x 4,0 mm precolumn (Merck, Darmstadt, Germany). Chromatographic methods were isocratic, except in the case of omeprazole when a linear gradient elution from 15 % A 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 the incubation and analysis conditions are summarized in table 1.

Diuron 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 diuron dilutions from stock solution of DMSO were used for each assay. The final amount of DMSO was 1% in incubation mixtures. For chlorzoxazone 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 [DDAB] in chloroform (100 mg/ml) to 200 μl L-α-phosphatidylethanolamine dioleyl (C18:1 [cis]9-) (PtdEta) 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 minutes by using 50% power.

The enzyme activities in the presence of diuron were compared with the control incubations into which only solvent were 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 MicroCal Origin 6.0 (MicroCal Software, Inc., Northampton, MA).

Correlation with model CYP 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 CYP substrate activities. A correlation was performed between the formation of N-demethyldiuron and each CYP 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 mean of duplicate incubations were used. Bivariate linear Pearson's correlation coefficients (r) were calculated between metabolite formations and model activities in livers.

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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-TOF-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, 3-(4-chlorophenyl)methylurea, 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 Figure1.

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, while 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, 100 µM were analyzed by LC-MS-MS. The results are presented in Figure 2.

A concentration of 100 µM of 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), while 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 homogenate 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 notice 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, while 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 samples 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 microsomal samples. N-demethyldiuron was quantified by triple quadrupole mass spectrometer as a result of diuron metabolism by seven mammalian microsomes including human. Diuron biotransformations by mammalian liver microsomes followed Michaelis-Menten kinetics as demonstrated by Eadie-Hofstee plots *(V versus V/S*). N-demethyldiuron formations were catalyzed by one or more microsomal enzymes with similar kinetics (data not illustrated). The kinetic parameters for the diuron metabolism in liver microsomes were determined by using a wide concentration range (1 µM - 400 µM) of diuron. The results are presented in Table 2.

K_m values in HLM, PLM and RabLM were generally similar (13.2, 14.4 and 15.6 µM), while the V_{max} in RabLM was 2-fold higher than those observed with PLM and HLM (4.9 nmol N-demethyldiuron/(mg protein*min)). Both DLM and MonLM had roughly similar K_m

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(31.4 and 37.9 μM) and V_{max} values (12,6 and 12.4 nmol N-demethyldiuron/(mg protein*min), respectively).

The intrinsic clearance (V_{max}/K_m) value for DLM (401 μl/(mg protein*min)) was higher than those for other mammalian liver microsomes, whereas RLM had the lowest (75 μl/(mg protein*min)). The HLM intrinsic clearance value was 174 μl/(mg 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 CYPs responsible for diuron metabolism. A screen of 11 human recombinant CYPs (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5) showed that all tested CYPs had detectable diuron N-demethylation activities. The kinetic parameters for the rCYPs 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_{max} values for CYP1A1 and CYP1A2 were the highest (215.6 and 173.4), while the V_{max} of CYP2C8 was the lowest (1.07 nmol N-demethyldiuron/ (nmol P450*min)). V_{max}/K_m values illustrated that CYP1A1, CYP1A2 and CYP2C19 were the most efficient rCYPs 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 CYPs [(1A2, 52); (2A6, 36); (2B6, 11); (2C8, 24); (2C9, 73); (2C19, 14); (2D6, 8); (2E1, 61) and (3A4, 111) pmol/mg microsomal protein] (Rostami-Hodjegan et al., 2007) and the actual intrinsic clearance values for various CYPs, the relative contributions of each CYP 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. about a 20-fold variation, and the mean value was 1.05 nmol N-demethyldiuron / (mg protein*min) (Table 4). The specific activities of CYP isoform-catalyzed reactions in microsomes from ten 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 = -0.02) of 0.67. With all other CYPs the correlations were less than 0.35.

The inhibitory interactions with different human liver CYPs. The effects of diuron on CYP-selective activities were determined in human liver microsomes. The only significant IC_{50} values were 4.0 μM for 7-ethoxycoumarin-*O*-deethylation (multiple CYPs) and 4.1 μM for 7-ethoxyresorufin-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.

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DISCUSSION

Diuron has been reported to be metabolized *in vivo* in rats, dogs (Hodge et al., 1967) and humans (Van Boven et al., 1990; Verheij et al., 1989) 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 mammalian hepatic microsomes and human liver homogenates was demethylation at the terminal nitrogen-atom, even if screening of the potential metabolites had been carried out 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 employed 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 [-N(CH3)CH2OH], followed by further oxidation to the formamide [-N(CH3)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 (CI) spectrum using LC/MS with a moving-belt interface, even though it was poorly separated from the didemethylation metabolite and its amount was small. However, in our study, the hydroxylated metabolites were not detected in any species even though they were searched from extracted mass chromatograms. If N-demethylation proceeds via the N-hydroxymethyl intermediate, its 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 concentrations 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 which 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-demethyldiuron at least in dogs.

An Animal to human efficiency comparison revealed that DLM metabolized diuron 2.3-fold more than HLM, while the rat had the smallest value (0.43-fold). Differences in efficiencies between rat, minipig and human liver microsomes have been reported by Lang et al. (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-demethyldiuron formation as a function of time correlated with the variation in kinetic parameters. DLM was the most active mammalian microsomes in diuron biotransformation over the time and concentrations used. However, in the 5 and 25 μM diuron incubations, N-demethyldiuron 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 was 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 CYPs were able to metabolize diuron to N-demethyldiuron. Kinetic characterization showed that diuron metabolism to N-demethyldiuron 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-demethyldiuron formation. Based on intrinsic clearance values and human hepatic microsomal P450 isoenzyme concentrations, the relative contribution of CYP1A2 to diuron N-demethylation was estimated to be 60% due to 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 > CYP2A6, CYP2C9 > CYP2E1 > 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 CYP isoform-catalyzed reactions in microsomes from ten human livers displayed high variability. An individual with the highest levels of both CYP1A2 and CYP2C19 (HL28) had the highest N-demethyldiuron formation, and the reverse was true for an individual (HL31) who had the lowest levels of both isoforms. 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.

An extensive *in vitro* screening of potential CYP-based interactions indicated that diuron inhibited CYP1A1/2 enzymes in HLM relatively potently, with an IC_{50} value of 4.1 μM. The corresponding values were higher than 100 μM for the other tested CYPs. It is not surprising that diuron inhibited CYP1A1/2, since these were CYP isoforms with a relatively

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low *Km* value for diuron N-demethylation. Very little has been reported about the potential interaction of diuron with other CYP enzyme activities. Diuron did not affect the CYP19-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 (NOEAL) 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 $10^{0.6}$ (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 $10^{0.5}$ (3.16)) (WHO/IPCS, 2005). In our results the highest uncertainty factor for animal to human differences in toxicokinetics (AK_{UF}) was 2.3-fold. In addition the uncertainty factor for human variability in toxicokinetics (HK_{UF}) was 1.5-fold, as defined by Renwick and Lazarus (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.

In order to obtain quantitative toxicokinetic data for comparison between individuals or between animals and human, human data are needed (Falk-Filipsson et al., in press). Moreover, risk assessment has to be carried out using quantitative chemical-specific data which 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, kinetic and interactions studies showed that the diuron biotransformation pathway involved the 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 rCYPs catalyzed diuron N-demethylation. The most important human liver CYPs were CYP1A2, CYP2C19 and CYP3A4. Diuron was a relatively potent inhibitor of human CYP1A1/2, which might be of significance at least in those occupational situations where workers are exposured 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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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. The overall scheme of the diuron metabolites detected in postmortem and hospitalized cases (Verheij et al., 1989; Van Boven et al., 1990). In addition, the exact and calculated masses of diuron and N-demethyldiuron detected in the current study are shown. In the postmortem case (full line metabolic pathways) DCPMU was the major metabolite. In the hospitalized case (discontinuous line pathways) DCPU was the major metabolite in blood, while DCPMU was dominant in urine.

Fig. 2. Diuron and N-demethyldiuron concentrations in the presence of different mammalian liver microsomes and human liver homogenate. Columns represent the mean of three separate determinations and the error bars represent the SD.

Table 1 Incubation and analysis conditions for inhibition assays in vitro. *

CYP	Substrate	Reaction	Conc. in incubation	Protein (mg/ml)	Cofactor system	Incubation Time (min)	Determination, λ (nm)	Eluent	reference
Multiple CYPs	7-Ethoxycoumarin	O-deethylation	10 µM	0.2	NADPH-regenerating	10	Fluorometric, 365/454		Greenlee and Poland, 1978
CYP1A1/2	7-Ethoxyresorufin	O-deethylation	1 µM	0.1	NADPH-regenerating	5	Fluorometric, 530/585		Burke et al., 1977
CYP2A6	Coumarin	7-hydroxylation	10 µM	0.2	NADPH-regenerating	10	Fluorometric, 365/454		Raunio et al., 1990
CYP2B	Pentoxyresorufin	O-deethylation	1 µM	0.1	NADPH-regenerating	5	Fluorometric, 530/585		Burke et al., 1977
CYP2B6	Bupropion	Hydroxylation	50 µM	0.4	NADPH	15	UV-HPLC, 214/204	75 % 50 mM *o*-phosphoric acid-buffer : 25 % acetonitrile	Turpeinen et al., 2004
CYP2C8	Amodiaquine	N-deethylation	30 µM	0.5	NADPH	20	UV-HPLC, 342	70 % 50 mM *o*-phosphoric acid-buffer : 30 % acetonitrile	Li et al., 2002
CYP2C9	Tolbutamide	Methylhydroxyl-ation	200 µM	0.15	NADPH-regenerating	20	UV-HPLC, 236/204	70 % 50 mM *o*-phosphoric acid-buffer : 30 % acetonitrile	Sullivan-Klose et al., 1996
CYP2C19	Omeprazole	5-hydroxylation	40 µM	0.1	NADPH	20	UV-HPLC, 304/204	25 mM *o*-phosphoric acid-buffer (A) and acetonitrile (B)	Äbelö et al., 2000
CYP2D6	Dextromethorphan	O-demethylation	10 µM	0.1	NADPH-regenerating	20	UV-HPLC, 204/280	75 % 50 mM *o*-phosphoric acid-buffer : 25 % acetonitrile	Kronbach et al., 1987
CYP2E1	Chlorzoxazone	6-hydroxylation	100 µM	0.1	NADPH-regenerating	20	UV-HPLC, 282/204	70 % 50 mM *o*-phosphoric acid-buffer : 30 % acetonitrile	Peter et al., 1990
CYP3A4	Midazolam	1`-Hydroxylation	10 µM	0.1	NADPH-regenerating	5	UV-HPLC, 245/204	60 % water : 40 % acetonitrile	Kronbach et al., 1989
CYP3A4	Omeprazole	Sulfoxidation	40 µM	0.1	NADPH	20	UV-HPLC, 304/204	25 mM *o*-phosphoric acid-buffer (A) and acetonitrile (B)	Äbelö et al., 2000

* Modified and updated from Abass et al., (2007b).

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Table 2: Kinetic parameters of N-demethyldiuron formations obtained with different mammalian liver microsomes[1].

Liver microsomes	V_{max} nmol/(mg protein * min)	K_m µM	CL_{int} µl/(mg protein * min)	AK_{UF}[2]
Human (HLM)	2.3 ± 0.1	13.2 ± 2.8	174.2	
Rat (RLM)	4.3 ± 0.2	57.6 ± 11.5	74.7	0.43
Mouse (MLM)	5.5 ± 0.2	25.7 ± 4.1	214.0	1.23
Dog (DLM)	12.6 ± 0.6	31.4 ± 6.9	401.3	2.30
Monkey (MonLM)	12.4 ± 0.7	37.9 ± 9.7	327.2	1.88
Minipig (PLM)	2.3 ± 0.1	14.4 ± 2.6	159.7	0.92
Rabbit (RabLM)	4.9 ± 0.1	15.6 ± 2.2	314.1	1.80

[1] Each value represents the mean ± std. error of three determinations.

[2] AK_{UF} Uncertainty factor for animal to human differences in toxicokinetics (WHO/IPCS, 2005).

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Table 3: Kinetic parameters of N-demethyldiuron formations obtained with recombinant CYPs[1].

Cytochrome P450 isoforms	V_{max} nmol / (nmol P450 * min)	K_m (µM)	CL_{int} µl / (nmol P450 * min)	Relative contribution (%)[2]
1A1	215.6 ± 18.8	90.0 ± 25.6	2395.6	N.D
1A2	173.4 ± 11.6	88.1 ± 19.5	1968.2	60.3
2A6	1.24 ± 0.1	143.5 ± 58.4	8.6	0.20
2B6	33.4 ± 1.5	53.8 ± 9.9	620.8	4.0
2C8	1.07 ± 0.1	173.6 ± 53.1	6.2	0.1
2C9	2.22 ± 0.2	46.9 ± 22.1	47.3	2.0
2C19	50.9 ± 2.2	30.0 ± 6.6	1696.7	14.0
2D6	64.9 ± 2.6	69.3 ± 10.3	936.5	4.4
2E1	1.93 ± 0.3	45.0 ± 25.1	42.9	1.5
3A4	21.1 ± 1.6	103.1 ± 24.8	204.7	13.4
3A5	5.94 ± 0.7	117.9 ± 40.4	50.4	N.D

N.D., not determined

[1] V_{max} and K_m values are represents the mean ± std. error of three determinations.

[2] Average human hepatic microsomal protein amounts of CYP enzymes are taken from Rostami-Hodjegan and Tucker (Rostami-Hodjegan et al., 2007).

Table 4: The specific activities of CYP isoform-catalyzed reactions in microsomes from ten human livers and their correlation with N-demethyldiuron formation[1].

Human Liver	CYP1A2	CYP2A6	CYP2B6	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4[2]	CYP3A4[3]	N-demethyldiuron nmol / (mg protein * min)
HL20	85.9	95.8	54.2	268.4	139.9	42.4	229.6	386.4	1174.9	17.2	1.33
HL21	31.5	465.5	77.2	219.2	128.4	21.7	215.3	686.7	772.9	30.2	0.66
HL22	31.1	323.8	118.2	221.9	103.2	105.7	194.2	314.4	3142.7	114.3	0.62
HL23	59.5	123.1	42.6	229.6	148.2	36.3	213.0	269.3	611.3	38.2	1.50
HL24	57.3	110.6	102.6	215.9	79.8	58.3	107.2	254.2	1034.4	30.2	1.26
HL28	109.8	549.2	4.5	332.7	139.3	124.0	154.1	773.5	4568.2	130.5	1.57
HL29	40.6	464.5	220.5	362.4	269.3	46.7	187.5	438.2	1760.6	43.7	1.45
HL30	61.7	147.0	40.9	209.3	98.2	45.4	67.9	244.7	813.7	57.7	0.76
HL31	3.8	225.3	35.5	152.6	178.9	-	88.9	471.2	511.6	12.0	0.08
HL32	18.9	311.7	35.1	175.6	201.0	21.4	111.2	136.0	602.5	20.5	1.24
Mean:	50.0	281.6	73.1	238.7	148.6	55.8	156.9	397.4	1499.3	49.5	1.05
Correlation coefficient (*r*):	**0.67**	0.08	0.10	**0.67**	0.20	0.07	0.35	-0.04	0.28	0.16	
HK_{UF}[4]											1.50

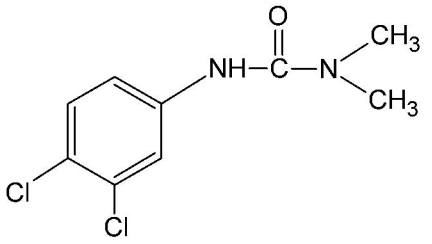
[1]Respective activities for CYPs presented as pmol min^{-1} mg^{-1} microsomal protein. Each presented value is an average of two parallel incubations.

[2] Midazolam α-hydroxyaltion,

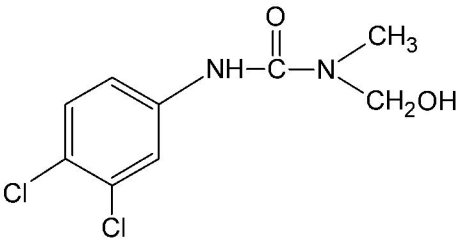
[3] Omeprazole sulphoxidation.

[4] HK_{UF} Uncertainty factor for human variability in toxicokinetics, (WHO/IPCS, 2005).

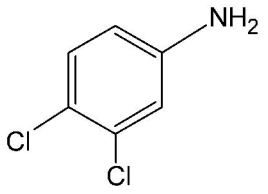
FIGURE 1



Diuron
3-(3,4-dichlorophenyl)-1,1-dimethyl urea
LC/MS-ESI+, MH^+=233Da
Exact mass: 233.0277, Calc. mass: 233.0248

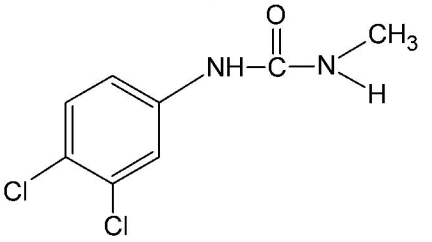


N-hydroxymethyldiuron
3-(3,4-dichlorophenyl)-1-hydroxymethyl-1- methyl urea

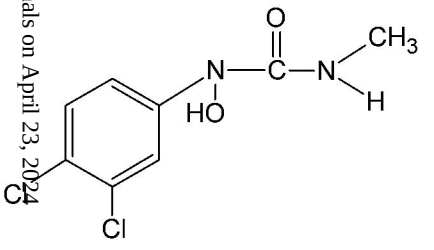


3,4-dichloroaniline

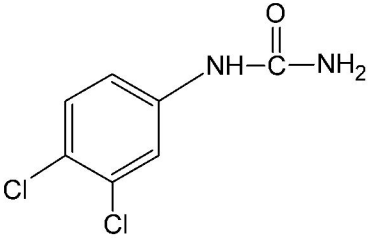
Cytochrome P450s



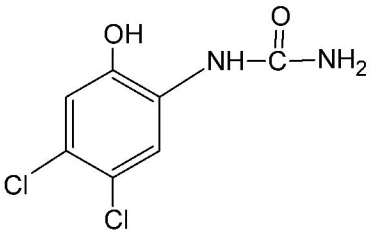
N-demethyldiuron (DCPMU)
3-(3,4-dichlorophenyl)-1-methyl urea
LC/MS-ESI+, MH^+=219Da
Exact mass: 219.0109, Calc. mass: 219.0092



N-hydroxy DCPMU
3-(3,4-dichlorophenyl)-3-hydroxy-1-methyl urea



N-didemethyldiuron (DCPU)
3,4-dichlorophenyl urea



6-hydroxy-3,4-dichlorophenyl urea

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FIGURE 2