Human hepatic cytochrome P450-specific metabolism of the organophosphorus pesticides methyl parathion and diazinon

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Abstract

Organophosphorus pesticides (OPs) are a public health concern due to their worldwide use and documented human exposures. Phosphorothioate OPs are metabolized by cytochrome P450s (CYPs) through either a dearylation reaction to form an inactive metabolite, or through a desulfuration reaction to form an active oxon metabolite, which is a potent cholinesterase inhibitor. This study investigated the rate of desulfuration (activation) and dearylation (detoxification) of methyl parathion and diazinon in human liver microsomes. In addition, recombinant human CYPs were utilized to determine the CYP-specific kinetic parameters ($K_m$ and $V_{max}$) for each compound for future use in refining human physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) models of OP exposure. The primary enzymes involved in bioactivation of methyl parathion were CYP2B6 ($K_m = 1.25 \mu M; V_{max} = 9.78 \text{ nmol/min/nmol P450}$), CYP2C19 ($K_m = 1.03 \mu M; V_{max} = 4.67 \text{ nmol/min/nmol P450}$) and CYP1A2 ($K_m = 1.96 \mu M; V_{max} = 5.14 \text{ nmol/min/nmol P450}$), the bioactivation of diazinon was mediated primarily by CYP1A1 ($K_m = 3.05 \mu M; V_{max} = 2.35 \text{ nmol/min/nmol P450}$), CYP2C19 ($K_m = 7.74 \mu M; V_{max} = 4.14 \text{ nmol/min/nmol P450}$) and CYP2B6 ($K_m = 14.83 \mu M; V_{max} = 5.44 \text{ nmol/min/nmol P450}$). CYP mediated detoxification of methyl parathion only occurred to a limited extent with CYP1A2 ($K_m = 16.8 \mu M; V_{max} = 1.38 \text{ nmol/min/nmol P450}$) and 3A4 ($K_m = 104 \mu M; V_{max} = 5.15 \text{ nmol/min/nmol P450}$), while the major enzyme involved in diazinon detoxification was CYP2C19 ($K_m = 5.04 \mu M; V_{max} = 5.58 \text{ nmol/min/nmol P450}$). The OP- and CYP-specific kinetic values will be helpful for future use in refining human PBPK/PD models of OP exposure.
Introduction

Organophosphorus pesticides (OPs) continue to be a human health concern due to their worldwide use, documented human exposures and neurotoxic potential (Jaga and Dharmani, 2006; Farahat et al., 2010; Farahat et al., 2011). Phosphorothioate OPs require metabolic activation to significantly inhibit acetylcholinesterase, which is thought to mediate the acute toxicity of these compounds (Myers et al., 1952; Sultatos, 1994). Metabolism studies for a variety of OPs have clearly indicated that their bioactivation is attributable to cytochrome P450 (CYP) mediated metabolism (Buratti et al., 2003; Sams et al., 2004; Foxenberg et al., 2007). Upon entry into the body, phosphorothioate OPs undergo a CYP mediated desulfuration reaction to form an active, highly toxic oxon intermediate metabolite which is responsible for the inhibition of acetylcholinesterase, butyrylcholinesterase and carboxylesterase (Ma and Chambers, 1994). Detoxification of the active oxon metabolite occurs by enzymatic hydrolysis mediated by A-esterases such as paraoxonase 1 (Pond et al., 1998). The parent OP compound can also undergo a CYP mediated dearylation reaction to form detoxified metabolites (Ma and Chambers, 1994). The balance between activation and detoxification of OPs determines their relative risk to humans.

Methyl parathion and diazinon are currently used in agriculture. Diazinon was once widely used in the USA for residential and garden applications, but since 2004 its use has been restricted to agriculture applications. Methyl paraoxon and diazoxon are the activated forms of methyl parathion and diazinon, respectively, whereas p-nitrophenol (PNP) and pyrimidinol (IMP) represent the detoxified metabolites.
PBPK/PD models allow researchers to predict the kinetics of absorption, distribution, metabolism, and excretion of OPs and also to assess the risks associated with their exposure using biochemical and physiological parameters generated from *in vitro* and *in vivo* studies in animals and in man (Knaak et al., 2004). Previously published PBPK/PD models have used kinetic parameters for OP metabolism largely generated from non-human studies. These models tend to be unstable, non-reproducible, and under-representative of interindividual variability when applied to humans (Knaak et al., 2004). To improve on existing models, there is a need to generate kinetic constants for human derived enzymes involved in metabolism of specific OPs. The use of species specific kinetic parameter values such as enzyme $K_m$ and $V_{max}$ values for the metabolism of OPs by specific recombinant human CYPs together with CYP-specific content (pmol of CYP/mg of microsomal protein) would allow for more accurate adjustments of model parameters for age, sex, genetic polymorphisms and other factors, which may influence CYP content and activity, and therefore OP metabolism and effects (Foxenberg et al., 2011).

There is currently a lack of human CYP-specific kinetic data for the metabolism of methyl parathion and diazinon. To date, no studies have identified the major human CYPs involved in methyl parathion metabolism. Studies which have assessed the human CYP-specific metabolism of diazinon have used relatively few substrate concentrations thus preventing the determination of kinetic parameters ($K_m$, $V_{max}$ and $Cl_{int}$) (Kappers et al., 2001; Sams et al., 2004; Mutch and Williams, 2006). The goal of the current study was to identify the human CYPs involved in methyl parathion and diazinon metabolism, as well as their respective $K_m$ and $V_{max}$ values for activation and detoxification. These human CYP-specific kinetic parameters can then be used in CYP based/age-specific PBPK/PD models for assessing the risk of OP exposure in man.
Methods

**Chemicals:** Methyl parathion (CAS 298-00-0), diazinon (CAS 333-41-5), methyl paraoxon (CAS 950-35-6), diazoxon (CAS 962-58-3) and IMP (CAS 2814-20-2) were purchased from ChemService Inc (West Chester, PA); p-nitrophenol (CAS 100-02-7) and tetraisopropyl pyrophosphoramide (iso-OMPA; CAS 513-00-8) were purchased from Sigma-Aldrich (St Louis, MO); MgCl₂ and EDTA were purchased from JT Baker (Phillipsburg, NJ) and were of at least reagent grade quality. Methanol and acetonitrile (EMD Chemicals; Gibbstown, NJ) were HPLC grade. Pooled human liver microsomes (HLM) and recombinant human CYPs (1A1, 1A2, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7) were purchased from Gentest (BD Biosciences; Bedford, MA). Recombinant CYP enzymes were prepared from a baculovirus-infected insect cell system containing oxidoreductase.

**Experimental conditions:** OP stock solutions were prepared in 50% methanol/water and stored at -20°C when not in use. Incubations with either HLM (0.5 to 1.0 mg protein/ml) or recombinant CYPs (0.03 to 0.06 nmol P450/0.5 ml) were carried out in buffer (100mM Tris-HCL, 5mM MgCl₂, 1mM EDTA and 50µM iso-OMPA; pH 7.4) at 37°C in a final volume of 0.25ml (methyl parathion) or 0.5ml (diazinon). Reactions were initiated with the addition of 1mM NADPH and incubated for 2 minutes at 37°C. EDTA and iso-OMPA were included to inhibit A-esterases and B-esterases, respectively (Reiner et al., 1993). The reaction was quenched with 1 volume of ice-cold methanol containing 0.1% phosphoric acid, centrifuged, and the supernatant was transferred to HPLC vials for analysis.

**Metabolite detection:** OPs and their respective metabolites were analyzed by reverse-phase HPLC (C18, 5µM particle size, 25cm x 4.6mm I.D; Supelco; St Louis, MO) utilizing a Hewlett-Packard Model 1100 HPLC with Model 1046A diode-array detector (Santa Clarita, CA).
Methanol (solvent A), 94.8% water/ 5% methanol/ 0.2% phosphoric acid (solvent B), acetonitrile (solvent C) and 94.9% water/ 5% methanol/ 0.1% phosphoric acid (solvent D) were utilized for gradient elution. For methyl parathion, the mobile phase consisted of: 0-6 min, 30% solvent A/70% solvent B; 6-20 min, linear gradient to 90% solvent A/10% solvent B; 20-23 min, 90% solvent A/10% solvent B; 23-24 min, linear gradient to 100% solvent A; 24-30 min, 100% solvent A; 30-33 min linear gradient to 30% solvent A/70% solvent B. For diazinon, the mobile phase consisted of: 0-5 min, 100% solvent D; 5-13 min, linear gradient to 40% solvent C/60% solvent D; 13-15 min, linear gradient to 45% solvent C/55% solvent D; 15-23 min, linear gradient to 100% solvent C; 23-28 min, 100% solvent C; 28-32 min, linear gradient to 100% D. The flow rate was 1 ml/min and the injection volume was 50µl. Methyl parathion and methyl paraoxon were detected at 275nm, PNP was detected as 320nm, diazinon and diazoxon were detected at 245m, and IMP was detected at 230nm. The retention times for methyl parathion, methyl paraoxon and PNP were 20.4, 15.4 and 13.7 minutes, respectively. The retention times for diazinon, diazoxon and IMP were 24.6, 20.0 and 11.6 minutes, respectively.

*Data analysis:* The kinetic values, $K_m$ and $V_{max}$, were determined by nonlinear regression analysis (Enzyme Kinetics module of SigmaPlot (SyStat Software Inc, V11)) of hyperbolic plots (i.e., velocity vs [S]) obeying Michaelis-Menten kinetics. OP parent compound concentration ($\mu$M) was set as the independent variable while rate of product formation (pmol/mg protein/min for HLMs or pmol/nmol P450/min for recombinant CYPs) was the dependent variable.

**Results**

Pooled HLM were utilized to determine kinetic values ($K_m$ and $V_{max}$) for metabolism of methyl parathion and diazinon. For methyl parathion, the $K_m$ and $V_{max}$ values were 0.99 $\mu$M and 0.11 nmol/min/mg protein for PNP formation and 66.8 $\mu$M and 1.84 nmol/min/mg protein for
methyl paraoxon formation. For diazinon, the $K_m$ and $V_{max}$ values were 31 µM and 1.18 nmol/min/mg protein for IMP formation and 30 µM and 0.73 nmol/min/mg protein for diazoxon formation.

Recombinant human CYPs were utilized to identify the human CYPs responsible for methyl parathion and diazinon metabolism. The Michaelis-Menten plots for the metabolism of methyl parathion by CYPs 2B6, 2C19 and 3A4 are shown in Figure 1 while Table 1 shows the kinetic parameters ($K_m$, $V_{max}$, $Cl_{int}$) for each human CYP capable of metabolizing methyl parathion and diazinon. The desulfuration (activation) of methyl parathion to methyl paraoxon was catalyzed primarily by CYP2B6 > CYP2C19 > CYP1A2, while the desulfuration (activation) of diazinon to diazoxon was catalyzed by CYP1A1 > CYP2C19 > CYP2B6 (Table 1). CYP3A4 had among the largest $V_{max}$ values for desulfuration of methyl parathion ($V_{max} = 9.78$ nmol/min/nmol P450) and diazinon ($V_{max} = 5.44$ nmol/min/nmol P450), but at the same time it also had the highest $K_m$ value (methyl parathion = 107 µM; diazinon = 28.7 µM) among the CYPs tested (Table 1). The $K_m$ values for desulfuration of methyl parathion by CYP2B6 ($K_m = 1.25$ µM) and CYP2C19 ($K_m = 1.03$ µM) were the lowest among the CYPs involved in methyl parathion metabolism (Table 1). For diazinon, the lowest $K_m$ values for desulfuration were for CYP1A1 ($K_m = 3.05$ µM) and CYP2C19 ($K_m = 7.74$ µM) (Table 1).

CYP mediated dearylation (detoxification) of methyl parathion only occurred to a limited extent with CYP1A2 ($Cl_{int} = 0.08$ ml/(nmol P450*min)) and CYP3A4 ($Cl_{int} = 0.05$ ml/(nmol P450*min)) while CYP2C19 was the major enzyme involved in diazinon detoxification (Table 1). The $K_m$ and $V_{max}$ for CYP2C19 dearylation of diazinon was 5.0 µM and 5.58 nmol/min/nmol P450 (Table 1).
Discussion

Hepatic microsomes contain many forms of CYPs making them ideal for assessing combinatorial CYP-mediated metabolism of OPs. While pooled HLM are useful to address metabolic capacity in the general sense, they do not provide information on the individual capacity of CYPs to metabolize an OP. To better address the limitations of using pooled HLMs, metabolism studies were conducted with ten recombinant human CYPs to identify the human CYPs responsible for methyl parathion and diazinon metabolism. CYP3A4 had among the largest $V_{\text{max}}$ values for desulfuration of methyl parathion and diazinon, but at the same time it also had the highest $K_m$ value among the CYPs tested, which minimizes the role of CYP3A4 in metabolism at lower OP exposures. This observation is consistent with previous studies where CYP3A4 was suggested to be important in the metabolism of OPs at higher concentrations (Buratti et al., 2003). CYP2B6 and CYP2C19 had the lowest $K_m$ values for desulfuration (activation) of methyl parathion, supporting the major role these CYPs play in metabolism at low-level real-world exposures. For diazinon, CYP1A1 and CYP2C19 had the lowest $K_m$ values for desulfuration. The identification of CYP2C19 as a key enzyme involved in diazinon activation expands upon a previous study which reported CYP2C19 to have a low $K_m$ for diazinon metabolism (Kappers et al., 2001).

CYP2B6 and CYP2C19 have also been identified as the major CYPs involved in the metabolism of other OPs such as chlorpyrifos and parathion  (Foxenberg et al., 2007). With regard to the metabolism of chlorpyrifos to its active oxon metabolite (chlorpyrifos-oxon), CYP2B6 is the most active CYP enzyme as shown by its low $K_m$ (0.81 µM), high $V_{\text{max}}$ (12.54 nmol/min/nmol P450) and high $\text{Cl}_{\text{int}}$ (15.56 ml/(nmol P450*min)), thus demonstrating the importance of CYP2B6 in chlorpyrifos activation (Foxenberg et al., 2007). Conversely,
CYP2C19 has the highest \( \text{Cl}_{\text{int}} \) for the metabolism of chlorpyrifos to its detoxified metabolite, 3,4,5-trichloropyridinol, and thus plays an important role in chlorpyrifos detoxification. Similar to methyl parathion and diazinon metabolism, CYP3A4 displays a high \( V_{\text{max}} \) for chlorpyrifos and parathion metabolism (Foxenberg et al., 2007); however, CYP3A4 also has a relatively high \( K_{\text{m}} \) for these OPs which minimizes its role at lower OP concentrations.

When assessing the contribution of a CYP isoform to the total hepatic metabolism of an OP it is also important to consider the relative CYP abundance of each CYP isoform within the liver. While CYP2B6 and CYP2C19 are more catalytically active (as represented by \( \text{Cl}_{\text{int}} \)) than CYP3A4 towards methyl parathion and diazinon, their hepatic content is about eight to nine times lower than CYP3A4 content (Yeo et al., 2004) and thus, CYP3A4 may become important in overall hepatic metabolism due to its sheer abundance, even if its activity is lower than other CYPs. The tissue distribution of CYPs is also important when assessing OP metabolism.

CYP2B6, the most active CYP involved in the bioactivation of methyl parathion and other OPs, is also located in most regions of the human brain (Gervot et al., 1999) suggesting that brain metabolism of OPs may be important when determining toxicity. A small amount of oxon formed in the brain may have a greater impact on systemic toxicity than the greater amount formed at the liver, which may not reach the brain. Albores et al. (2001) showed that CYP2B mediated the activation of methyl parathion in rat brain extracts, thereby further highlighting the significance of the CYP2B family of enzymes in OP metabolism.

The very limited dearylation (detoxification) of methyl parathion is markedly different than the metabolism of other OPs which show similar efficiencies for desulfuration and dearylation (Sams et al., 2004; Foxenberg et al., 2007). Anderson et al. (1992) assessed the metabolism of methyl parathion by subcellular fractions of isolated rat hepatocytes and reported
a greater production of methyl paraoxon than PNP, which agrees with results obtained in the current study. However, Zhang et al. (1991) reported that metabolism of methyl parathion by rat livers perfused in situ results in more PNP formation than methyl paraoxon. With regard to diazinon metabolism, CYPs were able to mediate desulfuration and dearylation. The main CYP involved in diazinon metabolism, CYP2C19, demonstrated higher activity towards the formation of the detoxified metabolite compared to the bioactive metabolite which is in agreement with previous reports (Sams et al., 2004; Mutch and Williams, 2006).

Some human PBPK/PD models for OP exposure currently use kinetic data acquired from rat liver microsomes which does not reflect human enzymes (Poet et al., 2004). Recent work has shown how these PBPK/PD models can be converted from a rat microsome metabolism model to a human CYP-specific metabolism model (Foxenberg et al., 2011). The CYP-specific $V_{\text{max}}$ values (nmol/min/nmol P450) obtained in the present study can be converted to in vivo values ($\mu$moles/hr/kg bw) by multiplying the in vitro values by time (min/hr), CYP content (nmol/mg microsomal protein), microsomal protein (mg/g liver) and liver weight (g liver/kg bw) and then dividing the resultant by 1.0E3. Inclusion of CYP-specific kinetics into a human PBPK/PD model allows differences in human hepatic CYPs to be accounted for. In addition to being the active CYPs in methyl parathion and diazinon metabolism, substantial interindividual variability exists in CYP2B6 and CYP2C19 hepatic content. Human hepatic expression levels of CYP2B6 and CYP2C19 can vary by 100-fold and 20-fold, respectively (Ekins et al., 1998; Koukouritaki et al., 2004). Additionally, both CYP2B6 and CYP2C19 contain polymorphisms capable of affecting enzymatic activity (Zanger et al., 2008). By having a PBPK/PD model that utilizes CYP-specific parameters, interindividual differences in CYPs can more accurately be accounted for which will result in more accurate models for predicting effects from specific OP exposures.
Authorship Contributions

Participated in research design: Ellison, Tian, Knaak, Kostuniak, Olson

Conducted experiments: Ellison, Tian

Contributed new reagents or analytic tools: Tian

Performed data analysis: Ellison, Tian

Wrote or contributed to the writing of the manuscript: Ellison, Tian, Knaak, Kostuniak, Olson
References


Footnotes

This research was supported by the Environmental Protection Agency (EPA) Science to Achieve Results [Grant R-83068301]. Corie Ellison was supported by a Research Supplement to Promote Diversity in Health-Related Research from the National Institute of Environmental Health Sciences (NIEHS) [Grant ES016308-02S]. The content is solely the authors’ responsibility and does not necessarily represent official views of the US EPA or the NIEHS.
Figure legends

Figure 1. Kinetic plots for methyl parathion metabolism by recombinant human CYP2B6, CYP2C19 and CYP3A4. Values represent the mean ± S.E.M of 3 (CYP3A4) or 4 (CYP2B6 and CYP2C19) determinants.
### Table 1. Methyl parathion and diazinon metabolism by recombinant human CYPs

<table>
<thead>
<tr>
<th>CYP</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/nmol P450)</th>
<th>$Cl_{int}$ (ml/(nmol P450*min))</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/nmol P450)</th>
<th>$Cl_{int}$ (ml/(nmol P450*min))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1$^a$</td>
<td>13.5 ± 14.22</td>
<td>1.51 ± 0.40</td>
<td>0.11</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP1A2$^a$</td>
<td>1.96 ± 0.64</td>
<td>5.14 ± 0.27</td>
<td>2.62</td>
<td>16.8 ± 6.07</td>
<td>1.38 ± 0.14</td>
<td>0.08</td>
</tr>
<tr>
<td>CYP3A4$^a$</td>
<td>107 ± 29.4</td>
<td>9.78 ± 1.52</td>
<td>0.09</td>
<td>104 ± 39.2</td>
<td>5.15 ± 1.09</td>
<td>0.05</td>
</tr>
<tr>
<td>CYP2B6$^b$</td>
<td>1.25 ± 0.32</td>
<td>10.39 ± 0.37</td>
<td>8.30</td>
<td>141 ± 72.3</td>
<td>1.42 ± 0.45</td>
<td>0.01</td>
</tr>
<tr>
<td>CYP2C19$^b$</td>
<td>1.03 ± 0.25</td>
<td>4.67 ± 0.15</td>
<td>4.51</td>
<td>48.9 ± 26.6</td>
<td>0.62 ± 0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>CYP2D6$^a$</td>
<td>50.3 ± 8.36</td>
<td>5.82 ± 0.41</td>
<td>0.12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP2E1$^c$</td>
<td>54.4</td>
<td>2.79</td>
<td>0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP1A1$^b$</td>
<td>3.05±1.08</td>
<td>2.35±0.19</td>
<td>0.771</td>
<td>29.9±30.4</td>
<td>1.77±1.00</td>
<td>0.059</td>
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<tr>
<td>CYP1A2$^b$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>23.8±11.1</td>
<td>2.66±0.37</td>
<td>0.112</td>
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<tr>
<td>CYP3A4$^b$</td>
<td>28.7±27.3</td>
<td>5.44±1.43</td>
<td>0.19</td>
<td>43.7±10.4</td>
<td>10.00±0.93</td>
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<tr>
<td>CYP2B6$^b$</td>
<td>14.83±7.13</td>
<td>5.44±0.58</td>
<td>0.366</td>
<td>13.94±3.37</td>
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<td>CYP2C19$^b$</td>
<td>7.74±11.45</td>
<td>4.14±0.84</td>
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<td>5.04±0.94</td>
<td>5.58±0.19</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Values represent the mean ± S.E.M of $^a$n = 3 or $^b$n = 4 determinants or $^c$the mean of n = 2 determinants.
Figure 1