Comparative Metabolism of 1,2,3,3,3-Pentafluoropropene in Male and Female Mouse, Rat, Dog, and Human Liver Microsomes and Cytosol and in Male Rat Hepatocytes Via Oxidative Dehalogenation and Glutathione S-Conjugation Pathways

Xing Han, Bogdan Szostek, Ching-Hui Yang, Steve F Cheatham, Robert T Mingoia, Diane L Nabb, Shawn A Gannon, Matthew W Himmelstein, and Gary W Jepson

DuPont Haskell Global Centers for Health & Environmental Sciences (X.H., R.T.M., D.L.N., S.A.G., M.W.H., G.W.J.), DuPont Corporate Center for Analytical Sciences (B.S.), DuPont Pioneer (C.-H.Y.), and DuPont Crop Protection (S.F.C.), Newark, Delaware
Running title page

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b) Corresponding author:

Xing Han, Ph.D.

DuPont Haskell Global Centers for Health & Environmental Sciences

P.O. Box 50

1090 Elkton Road

Newark, DE 19714

U.S.A.

Phone: (302) 458-5808

Fax: (302) 451-3568

Email: xing.han@usa.dupont.com

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d) Nonstandard Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; FID, flame ionization detector; G6P, glucose-6-phosphate; GSH, glutathione; GWP, global warming potential; HFC-134a, 1,1,1,2-tetrafluoroethane; HFP, hexafluoropropene; GST, glutathione S-transferase; PCA, perchloric acid; PFP,
1,2,3,3-pentafluoropropene; PFPG, S-(1,2,3,3,3-pentafluoropropyl)-glutathione; PFPA, 3,3,3-trifluoropyruvaldehyde; TFPAA, 2,3,3,3-tetrafluoropropionic acid; TFP, S-(2,3,3,3-tetrafluoropropenyl)-glutathione; TPA, 2,3,3,3-tetrafluoropropionaldehyde
Abstract

In vitro metabolism of 1,2,3,3,3-pentafluoropropene (PFP) is investigated in the present study. PFP was metabolized via cytochrome P450-catalyzed oxidative dehalogenation in liver microsomes and glutathione S-transferase (GST)-catalyzed conjugation in liver microsomes and cytosol. Two oxidation products, 2,3,3,3-tetrafluoropropionaldehyde (TPA) and 3,3,3-trifluoropyruvaldehyde (TFPA), and two glutathione (GSH) conjugates, S-(2,3,3,3-tetrafluoropropenyl)-GSH (TFPG) and S-(1,2,3,3,3-pentafluoropropyl)-GSH (PFPG) were identified. Enzyme kinetic parameters for the formation of TFPA, TFPG, and PFPG were obtained in male and female rat, mouse, dog, and human liver microsomes and cytosol, and were confirmed using freshly isolated male rat hepatocytes. For the TFPA pathway, dog microsomes exhibited much larger $K_m$ values than rat, mouse, and human microsomes. Sex differences in the rates of metabolism within a given species were minor and generally less than two-fold. Across the species, liver microsomes were the primary subcellular fraction for GSH S-conjugation and the apparent reaction rates for the formation of TFPG were much greater than those for PFPG in liver microsomes. PFPG was unstable and had a half-life of about 3.9 h in a phosphate buffer (pH 7.4 and 37°C). The intrinsic clearance values for the formation of TFPA were much greater than those for the formation of GSH S-conjugates, suggesting that cytochrome P450-mediated oxidation is the primary pathway for the metabolism of PFP at relatively low PFP concentrations. Because saturation for GST-mediated reactions was not reached at the highest possible PFP concentration, GSH S-conjugation may become a much more important pathway at higher PFP concentrations (relative to the $K_m$ for TFPA).
Introduction

1,2,3,3,3-pentafluoropropene (PFP) belongs to a group of fluoroalkenes which have chemical and environmental properties that make them attractive alternatives to substances with high global warming potential. A number of fluoroalkenes are found to be nephrotoxic in rats, such as hexafluoropropene (Koob and Dekant, 1990), tetrafluoroethylene (Odum and Green, 1984), and 2-(fluoromethoxy)-1,1,3,3,3-pentafluoro-1-propene (compound A, Iyer and Anders, 1997). The mechanism is considered to involve a multi-step bioactivation pathway including hepatic glutathione (GSH) S-conjugation of the fluoroalkenes, followed by enzymatic hydrolysis of the GSH S-conjugates to cysteine S-conjugates, renal uptake of cysteine S-conjugates, and formation of reactive species via the renal cysteine S-conjugate β-lyase pathway (Anders and Dekant, 1998). Compared to the rat, the human is at a much less risk for fluoroalkene-induced nephrotoxicity (Anders and Dekant, 1998; Altuntas and Kharasch, 2001; 2002; Altuntas et al., 2003), due to the fact that the renal β-lyase activity in the human is much lower than that in the rat (Anders and Dekant, 1998; Altuntas and Kharasch, 2001; 2002; Altuntas et al., 2003).

In addition to GSH S-conjugation, cytochrome P450 (P450)-mediated oxidation is also a possible metabolic pathway for fluoroalkenes. This oxidative pathway was not observed for some perfluorinated alkenes, such as hexafluoropropene (Koob and Dekant, 1990) and tetrafluoroethylene (Odum and Green, 1984), but appears to be sensitive to the degree of fluorination, and is enhanced by chloro substitutions (Bolt et al., 1982; Baker et al., 1987).
In order to understand the relative importance of the two metabolic pathways for PFP in different species, we investigated the oxidation and GSH S-conjugation potentials of PFP in rat liver microsomes and cytosol and compared enzyme kinetics for the formation of one stable oxidation product and two GSH S-conjugates in male and female mouse, rat, dog, and human liver microsomes and cytosol. Freshly isolated male rat hepatocytes were used to confirm the metabolic pathways and enzyme kinetic parameters obtained in the liver subcellular fractions.

Materials and Methods

Materials. PFP (CAS# 2252-83-7, purity > 99.9%) was provided by DuPont Fluoroproducts. Its identity and purity were confirmed by NMR, GC-flame ionization detector (FID), and GC-mass selective detector methods. 3,3,3-Trifluoropyruvaldehyde (TFPA, CAS# 91944-47-7, purity > 99%) was purchased from Apollo Scientific (Bredbury, UK). 2,3,3,3-Tetrafluoropropanoic acid (TFPAA, CAS# 359-49-9, purity > 97%) was purchased from Oakwood Product (West Columbia, SC). Pooled liver microsomes and cytosol from rat, mouse, dog, and human were purchased from either In Vitro Technologies (Baltimore, MD) or Xeno Tech (Lenexa, KS). Cell culture media and buffers were obtained from Invitrogen (Carlsbad, CA). 2,4-Dinitrophenylhydrazine (DNPH) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals, if not specified in the article, were obtained from Sigma-Aldrich.

Animals. Male Crl:CD(SD)IGS BR rats were obtained from Charles River Laboratories (Raleigh, NC). Upon arrival, all animals were housed in quarantine for at least 4 days. Animals were provided tap water ad libitum and fed PMI® Nutrition International, LLC...
Certified Rodent LabDiet® 5002 ad libitum. At the time of hepatocyte isolation, rats were 6-8 weeks of age. Animal rooms were maintained at a temperature of 18-26°C and a relative humidity of 30-70%, and were artificially illuminated (fluorescent light) on a 12 h light/dark cycle.

**Rat Hepatocytes Isolation.** The procedures for isolation of rat hepatocytes were described previously (Nabb et al., 2006; Mingoia et al., 2007). The cells were suspended in Leibovitz L-15 medium at pH 7.4. Cells were counted in the presence of 0.04% trypan blue. The viabilities of the cells were approximately 90%.

**Phosphate Buffer-Air Partition of PFP.** PFP dose concentrations were prepared by diluting pure gaseous compound in Tedlar® bags (SKC Inc., Eighty Four, PA) containing known volumes of room air. Approximately 35 mL of known concentration PFP gas was purged through a 10-mL gas-tight glass vial that contained 2 mL 0.1 mM potassium phosphate buffer (pH 7.4). After incubation at 37°C for 2 h, 1 mL of the phosphate buffer was quickly transferred via an air-tight syringe to another PFP-free vial. The second vial was heated at 75°C for approximately 20 min. PFP concentration in the headspace of the second vial, which corresponds to the concentration of dissolved PFP in the phosphate buffer, was determined by a GC-FID method on an Agilent HP6890 instrument equipped with a Agilent HP-5 column (30m × 320 µm, 0.25 µm film thickness) and a Gerstel MP2 autosampler.

**Biosynthesis of S-(2,3,3,3-tetrafluoropropenyl)-glutathione (TFPG).** In a 40-mL airtight glass flask, male rat microsomes (2 mg protein/mL) were incubated at 37°C with pure PFP gas and 10 mM GSH in 10 mL of 0.1 M potassium phosphate buffer, pH 7.4, containing 1% ethanol. After 2 h incubation, the sample was concentrated using a MF
C18 Isolute SPE column (Biotage, Uppsala, Sweden) in acetonitrile:water (1:1). TFPG was purified by LC/MS method. The sample was evaporated to dryness on a speed-vacuum and re-dissolved in approximated 1 mL of CD$_3$CN/D$_2$O (2:8) for NMR analysis.

**Enzymatic Assay.** All enzymatic reactions were stopped by the addition of 10% volume of 35% perchloric acid (PCA) and with brief centrifugation to remove the protein precipitants.

For metabolite identification experiments, 1 mg/mL microsomes or cytosol from male rats were incubated for 1 h at 37°C with 120,000 ppm PFP in 0.1 M potassium phosphate buffer, pH 7.4, containing 10 mM GSH. The microsomal samples also contained a NADPH regenerating system consisting of 0.1 mM EDTA, 10 mM glucose-6-phosphate (G6P), 3.6 U/mL G6P dehydrogenase, 15 mM MgCl$_2$, and 0.525 mM NADP. After PCA precipitation and centrifugation, the samples were analyzed directly by LC/MS method. Aliquots of the microsomal samples were also mixed with equal volume of DNPH derivatization solution (0.3% DNPH in water/concentrated HCl/acetonitrile = 51:29:20) before analysis for the identification of ketone or aldehyde metabolites (Olson and Swarin, 1985; van Leeuwen et al., 2004).

The enzyme kinetics for the formation of TFPA were determined in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.5 mg/mL microsomal proteins, NADPH regenerating system, and varied PFP concentrations from 250 to 50,000 ppm. The reactions were equilibrated at 37°C for 10 min before being initiated with the addition of 0.525 mM (final concentration) of NADP, and were stopped 20 min after the initiation. The samples were mixed with equal volume of the DNPH solution for LC/MS quantification. TFPA standards were prepared in a heat-inactivated microsomal matrix.
that contained exact compositions of the microsomal reactions mentioned above except for the PFP dose, and were also derivatized with equal volume of the DNPH solution before quantification.

The enzyme kinetics for the formation of TFPG and PFPG were determined in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.2 mg/mL microsomal proteins or 0.5 mg/mL cytosol proteins and PFP concentrations ranging from 100,000 to 1,000,000 ppm. For spontaneous reactions, heat-inactivated microsomes or cytosol at the same protein concentrations was used instead. The reactions were equilibrated at 37°C for 10 min before being initiated with the addition of 10 mM (final concentration) of GSH, and were stopped 60 min after the initiation. Immediately after PCA precipitation and brief centrifugation to remove the precipitants, the samples were quickly chilled to 4°C and analyzed by LC/MS methods described in Sample Analysis. Biosynthesized TFPG was prepared in a heat-inactivated microsomal or cytosol matrix and was used as quantification standards for both TFPG and PFPG samples.

For enzymatic reactions in isolated rat hepatocytes, PFP at various concentrations were incubated with $2 \times 10^6$ cells/mL hepatocytes in the Leibovitz L-15 medium at pH 7.4 and 37°C for 20 (TFPA samples) or 60 min (TFPG and PFPG samples). The samples were analyzed the same way as described above.

**PFPG Stability.** In a 10-mL gas-tight glass vial, pure PFP gas was allowed to react with 10 mM GSH in 3 mL 0.1 mM potassium phosphate buffer at pH 7.4. After incubation at 37°C for 2 h, the phosphate buffer was transferred to another PFP-free vial that was pre-incubated at 37°C. The vial was loosely capped and aliquots of the buffer were taken at different time point over the incubation period. PFPG level at each time point was
determined by LC/MS method. Spontaneous degradation of PFPG in the buffer was
assumed following first-order kinetics:

\[
\ln C(t) = \ln C(0) - \left(\frac{0.693}{T_{1/2}}\right) t
\]

where \( t \) is incubation time in h; \( C(0) \) and \( C(t) \) are PFPG concentrations at time zero and \( t \), respectively; and \( T_{1/2} \) is PFPG half-life in h.

**Sample Analysis.** NMR data were acquired on a Bruker Avance 400MHz spectrometer
using a \(^{19}F\), \(^1H\), \(^{13}C\) TXO probe optimized for \(^{19}F\) detection. Fluorine TFPG spectra were
acquired in CD\(_3\)CN/D\(_2\)O (2:8) with a spectra window of 100KHz, an acquisition time of
1.3 seconds and 9431 scans. Probe calibrations for pulse width and signal-to-noise were
performed with 0.05\% \( \alpha,\alpha,\alpha\)-trifluorotoluene in C\(_6\)D\(_6\). Fluorine chemical shift was
calibrated using CFCl\(_3\). Each sample was tuned and matched before data acquisition.
Spectra were acquired at 27\(^\circ\)C. The concentration of the biosynthesized TFPG, which
was used as an analytical standard for the enzyme kinetic studies, was determined by
comparing the intensity of the CF\(_3\) resonance of TFPG (-69.8 ppm) against the CF\(_3\)
resonance of a set of TFPAA (-74.3 ppm) standards at known molar concentrations.

Samples for metabolite identification experiments were analyzed using two
LC/MS/MS systems. The first LC/MS/MS system included an Applied Biosystems 4000
QTrap mass spectrometer (Foster City, CA), an Agilent 1100 HPLC (Palo Alto, CA), and
a CTC PAL autosampler (LEAP technology, Carrboro, NC). Samples were eluted on an
Agilent Zorbax SB-C18 column (2.1 × 30 mm, 3.5 \( \mu \)m) by maintaining 98\% eluent A (2
mM ammonium acetate in water) for 0.5 min and with a linear gradient, changing to
100\% eluent B (methanol) within the next 13 min. The flow rate was kept at 0.3 mL/min.
Turbo Spray ionization in negative and positive ion mode was used for the QTrap and the
probe temperature was set at 450°C. The second LC/MS/MS system included Waters Acquity Ultra Performance LC and Q-Tof II mass spectrometer (Milford, MA). Samples were analyzed with the same LC conditions except that a 1.8 µm Zorbax column was used instead. The electrospray source conditions for the Q-Tof were 2.5 kV for the capillary, 35 V for the cone, 120 °C for the source temperature, and 350°C for the desolvation temperature.

Isolation of biosynthesized TFPG was conducted on an Agilent 1100 LC system and a Waters ZQ mass spectrometer. Samples were separated on a Zorbax SB-C18 column (2.1 × 150 mm, 5 µm) by maintaining 98% eluent A (0.1% formic acid in water) for 1 min and with a linear gradient, changing to 40% eluent B (0.1% formic acid in acetonitrile) within the next 4 min. The flow rate was kept at 0.5 mL/min.

Samples for enzyme kinetics measurements were analyzed on a Waters Quattro Micro mass spectrometer and a Waters 2795 HPLC (Milford, MA). DNPH-derivatized TFPA samples were separated on a Waters XTerra column (C18, 2.5 µm, 2.1 × 30 mm) within 6 min by linear gradient from 80% eluent A (2 mM ammonium acetate in water) to 80% eluent B (acetonitrile) at a flow rate of 0.3 mL/min. Data were acquired in negative ion mode with a capillary voltage at 3 kV, a cone voltage at 25 V, source temperature at 120°C, and desolvation temperature at 350°C by single ion recording (SIR) of the de-protonated molecular ion (M-H)- of 305 m/z.

The LC conditions for TFPG and PFPG samples were analogous to the method used for TFPG isolation. Data were acquired at positive ion mode with a capillary voltage at 3 kV, a cone voltage at 15 V, source temperature at 120 °C, and desolvation temperature.
temperature at 350 °C by single ion recording (SIR) of the protonated molecular ions (M+H)^+ of 420 m/z (TFPG) and 440 m/z (PFPG).

**Data Analysis.** The velocity *versus* substrate concentration, $V/[S]$, plots for metabolite TFPA were fitted to the Michaelis-Menten equation in software package Origin (version 7.0, OriginLab Corp., Northampton, MA) to obtain $V_{\text{max}}$ and $K_m$ values. Intrinsic clearance ($V_{\text{max}}/K_m$) values for the GSH S-conjugates were the average of the $V/[S]$ ratios at one to four different PFP concentrations that were low relative to the $K_m$. 
Results

Metabolite Identification. All the supporting figures for the metabolite identification of PFP were summarized in the Supplemental Figure 1 to Supplemental Figure 10. Figure 1 shows PFP metabolites identified in male rat liver microsomes in the presence of the NADPH regenerating system and GSH. In negative ion mode, DNPH-derivatized TFPA has a molecular ion of 485 m/z with major fragments of 465 (loss of HF), 445 (loss of 2HF), 302, 279, and 182 m/z. Its product ion spectrum and postulated fragments were present in S1 and S2, respectively. The retention time and the product ion spectrum of the TFPA metabolite matched that of the authentic standard (data not shown). DNPH-derivatized TPA has a molecular ion of 309 m/z with major fragments of 289 (loss of HF), 269 (loss of 2HF), 241, 231, 214, and 181 m/z. Its product ion spectrum and postulated fragments were present in S3 and S4, respectively. No authentic standard was available for this metabolite. There was a possibility that this metabolite was an isomeric ketone. However, the fragmentation pattern, especially multiple losses of HF to form ion m/z 269 or 241, suggests that the metabolite is an aldehyde. The rationale is mainly based on the findings that a similar type of fragmentation pattern was observed for 3,3,3-trifluoropropanal derivatized with DNPH, but not for DNPH-derivatized 1,1,1-trifluoroacetone (data not shown).

The product ion spectra and postulated structures of fragments ions for the two GSH S-conjugates, TFPG and PFPG, were present in S5 to S8. The molecular ions for TFPG and PFPG at negative ion mode were 418 and 438 m/z, respectively. Majority of the fragment ions observed in the spectra (m/z 179, 210, 254, 272) were originated from the fragmentation of the GSH moiety (Naylor et al., 1988). Proton decoupled and coupled
$^{19}$F-NMR spectra of a biosynthesized TFPG sample was shown in S9 and S10. The CF$_3$ and the single fluorine signals were at approximately -69.8 and -130.8 ppm, respectively. In both proton coupled and decoupled spectra the CF$_3$ group was a clean doublet. Also, the single fluorine signal does not appear to be geminally coupled to a proton. These data were consistent with the single fluorine position we proposed for TFPG. We could not obtain an acceptable PFPG NMR spectrum due to the stability issue of PFPG (see below). The structure of PFPG was proposed based on its molecular mass, the similarities between the mass spectra from TFPG and PFPG, and the addition mechanism for the formation of GSH S-conjugates of fluoroalkenes (Koob and Dekant, 1990; Anders and Dekant, 1998).

We also obtained the authentic standard for 2,3,3,3-tetrafluoropropanoic acid (TFPAA) and looked for possible existence of this metabolite in the hepatocyte samples that were used for the enzyme kinetic study. We did not find a corresponding peak in the hepatocyte samples at the retention time of the standard.

**Phosphate Buffer-Air Partition of PFP.** Partitioning of the gaseous compound PFP to 0.1 M potassium phosphate buffer (pH 7.4) was determined at 37°C. Figure 2 shows that concentrations of PFP in the buffer were proportional to the compound concentrations in the gas phase up to 1,000,000 ppm (the pure gas). The substrate concentrations of PFP in the enzyme kinetics studies, therefore, were calculated using 1.153 nM (in the buffer) per ppm in the gas phase. The effect of protein on the solubility of PFP in the buffer was considered negligible due to the finding that the metabolite formation was linear over a wide range of the protein concentrations from 0.1 up to 2 mg/mL (see below for details).
**Enzyme Kinetics for the Formation of TFPA in Liver Microsomes.** The velocities of TFPA formation in male rat liver microsomes were found to be linear to the microsomal protein concentrations between 0.1 and 1 mg/mL, and up to 30 min incubation time (data not shown). The representative $V/[S]$ curves of TFPA obtained in male rat, mouse, dog, and human liver microsomes (0.5 mg/mL) and 20 min incubation were shown in Figure 3. All of the $V/[S]$ curves were fitted to the Michaelis-Menten equation to obtain the enzyme kinetic parameters (Table 1). All species produced TFPA in their liver microsome samples. The order of the $V_{\text{max}}$ values was dog > human > mouse > rat. The sex differences were minor relative to the differences between the species. $K_m$ values were quite similar for rat, mouse, and human, and ranged from 1.33 to 2 µM. Dog had much larger $K_m$ values, ~61 µM for the male and ~88 µM for the female. As a result, intrinsic clearance rates ($V_{\text{max}}/K_m$) for the dog were the slowest compared to other species. Human in general had the fastest rate of intrinsic clearance via the oxidative pathway to TFPA, with the exception of the minor difference between female mouse and female human.

**Enzyme Kinetics for the Formation of GSH S-conjugates in Liver Microsomes and Cytosol.** Spontaneous, non-enzymatic GSH S-conjugations of PFP to form TFPG and PFPG were observed (Figure 4). The rates obtained in heat-inactivated microsomes and cytosol were lower than the rates from live microsomes and cytosol and roughly ranged from one-tenth to one-half of the enzymatic reactions (data not shown). The velocities of TFPG and PFPG formation were linear to the protein concentrations between 0.1 and 1 mg/mL for male rat microsomes and between 0.25 and 2 mg/mL for male rat cytosol within 60 min of incubation (data not shown). In experiments to determine the $K_m$, there...
was a linear increase in the velocities of TFPG and PFPG formation up to the highest possible substrate concentration in the gas phase (pure PFP gas at 1,000,000 ppm, Figure 4). The intrinsic clearance rates for PFP via the GSH S-conjugation pathway were determined by linear regression and the contribution from the enzymatic reactions were summarized in Table 2. For all species, TFPG was formed predominantly in the microsomal samples, whereas PFPG was formed at a relatively higher rate in the cytosol. TFPG, if judged by the combined rates of formation in microsomes and cytosol, was the major product for all species. The order of PFP clearance rates via TFPG formation in the microsomes was female rat ≈ male rat ≈ human ≈ dog ≈ mouse.

**Enzyme Kinetics for the Formation of TFPA, TFPG, and PFPG in Freshly Isolated Hepatocytes.** The parameters for the formation of TFPA, TFPG, and PFPG in male rat hepatocytes were summarized in Table 3. The intrinsic clearance value for TFPA pathway was over 500 times larger than the combined values for the GSH S-conjugates. For the two GSH S-conjugates, intrinsic clearance was approximately 5 times faster for the TFPG pathway than that for the PFPG pathway. The contributions of spontaneous reaction to the clearance rates of TFPG and PFPG were not characterized due to the lack of appropriate controls for the hepatocyte samples.

**PFPG Stability.** GSH S-conjugate PFPG was unstable, and underwent spontaneous degradation in both acetonitrile and aqueous solutions. Figure 5 shows the disappearance of PFPG as a function of time in 0.1 M potassium phosphate buffer at pH 7.4 and 37°C. The half-life of PFPG under such conditions was approximately 3.9 hours. No effort was made to identify the degradation product of PFPG.
Discussion

Comparative *in vitro* metabolism of PFP was investigated in this study. PFP underwent glutathione *S*-transferase (GST) and P450-mediated metabolism to form GSH *S*-conjugates TFPG and PFPG and oxidation products TFPA and TPA (Figure 1). Human and rat intrinsic clearance values for the formation of GSH *S*-conjugates were quite comparable and greater than the mouse and dog (Table 2). For the TFPA pathway, the dog exhibited somewhat different enzyme kinetics relative to the other species tested, the human had the highest intrinsic clearance values (Table 1). The differences between the genders were minor (Table 1 and 2).

We confirmed the results obtained from liver subcellular fractions with freshly isolated male rat hepatocytes. Both oxidation and conjugation pathways were observed in rat hepatocytes. Quantitatively, the intrinsic clearance value for TFPA pathway was approximately 3-times higher in the hepatocytes than that in the microsomes, mainly due to the 3-times smaller *K*ₘ value observed in the hepatocytes (Table 1 vs. Table 3). For the two GSH *S*-conjugates, the clearance values obtained from the hepatocytes were quite comparable to the values from liver subcellular fractions (Table 2 vs. Table 3). Overall, we’ve demonstrated that our combined microsomal and cytosol data were in good agreement with the results obtained from intact liver cells.

The mechanism of P450-mediated oxidation of halogenated alkenes was described previously (Testa, 1995) as the formation of a carbocation intermediate which collapsed to an epoxide and an aldehyde or ketone. The oxidation of PFP in liver microsomes is consistent with this mechanism. For PFP, the formation of the epoxide could be transient and readily hydrolyzed to the stable ketoaldehyde product TFPA via
CYP2E1 (Schuster et al., 2008). The aldehyde product TPA, as opposed to a ketone, was also identified in our study (Figure 1), which however, could be a product of direct P450 oxidation without bypassing an epoxide intermediate (Schuster et al., 2008). We did not find the downstream acid product TFPAA in our in vitro samples, but cannot rule out the possibility of this acid being formed in vivo.

In our study, both addition and addition-elimination mechanisms (Anders and Dekant, 1998) were observed in the GST-catalyzed reaction of PFP to form PFPG and TFPG, respectively. Across species, the reaction rates for the formation of TFPG were much greater than that for PFPG in liver microsomes. In contrast, PFPG was generally the preferred product in cytosol. This pattern agreed with the previous observation for hexafluoropropene (Koob and Dekant, 1990). Overall, TFPG was the major GSH S-conjugation product as confirmed in our hepatocyte experiments (Table 3). PFPG was quantified using TFPG as the analytical standard, primarily due to the stability issue of PFPG in the solvent (Figure 5). We do not anticipate huge difference in ionization efficiency between TFPG and PFPG, but it’s important to note that absolute quantification of PFPG was not achieved in this study. We’ve also observed that, at physiological conditions (pH 7.4 and 37°C), PFPG had a half-life of about 3.9 h (Figure 5). In contrast, TFPG was considerably more stable (data not shown). This observation, combined with our enzyme kinetic data for TFPG and PFPG, leads us to conclude that TFPG could be more physiologically and toxicologically relevant than PFPG.

Our results showed that intrinsic clearance of PFP via P450-catalyzed oxidation to form TFPA was much faster than the clearance from liver GST-catalyzed conjugation (Table 3), suggesting that the P450 mechanism is the primary pathway for the
metabolism of PFP. It’s important to note that the $K_m$ value for the formation of TFPA was quite small and for the most part, below 2 µM. This indicates that the GST pathway could become relatively more important at a higher substrate concentration. It can be estimated that in male rat hepatocytes, 260 µM of PFP would allow the formation of TFPG at a rate of 65 pmol/min/10^6 cells, the $V_{max}$ value for TFPA formation (Table 3). In addition, we observed spontaneous, non-enzymatic reaction between GSH and PFP (Figure 4), which could contribute significantly to the overall formation of GSH S-conjugates of PFP in the body.

It has been proposed that P450-mediated oxidation reactions of haloalkenes could form reactive intermediates that are hepatotoxic (Costa and Ivanetich, 1980; Bolt et al., 1982; Ortiz de Montellano et al., 1982; Baker et al., 1987; Yoshioka et al., 2002), and the GST-mediated GSH S-conjugation of haloalkenes could initiate a bioactivation pathway for nephrotoxicity (Odum and Green, 1984; Koob and Dekant, 1990; Lock and Ishmael, 1998). It was also suggested that the preference of P450 vs. GST-mediated pathways for the haloalkenes to some extent correlated with their specific toxicities (hepatotoxicity vs. nephrotoxicity) observed in vivo (Commandeur et al., 1987; Dekant et al., 1987). In this study, we confirmed both oxidation and GSH S-conjugation metabolism of PFP in vitro and obtained enzyme kinetic parameters for the formation of one oxidation product and two GSH S-conjugates in multiple animal species. The findings presented here provide a foundation for further study of the metabolism and toxicity of PFP in vivo.
Authorship Contributions

Participated in research design: Han, Szostek, Himmelstein, Jepson

Conducted experiments: Han, Szostek, Yang, Cheatham, Mingoia, Nabb, Gannon

Contributed new reagents and analytical tools: Szostek, Cheatham

Performed data analysis: Han, Yang, Cheatham

Wrote or contributed to the writing of the manuscript: Han, Szostek, Himmelstein
References


Nabb DL, Mingoia RT, Yang CH and Han X (2006) Comparison of basal level metabolic enzyme activities of freshly isolated hepatocytes from rainbow trout (Oncorhynchus mykiss) and rat. *Aquat Toxicol* **80**:52-59.


Figure Legends

Figure 1. PFP metabolites that were identified in male rat liver microsomes in the presence of NADPH regenerating system and GSH.

Figure 2. Phosphate buffer-air partition curve for PFP in 0.1 M potassium phosphate buffer at pH 7.4 and 37°C. Duplicate aqueous concentration determinations are shown for each gaseous concentration. The linear curve-fit was forced to intercept with the origin.

Figure 3. Representative $V/[S]$ plots for the formation of TFPA in male rat, mouse, dog, and human liver microsomes in the presence of NADPH regenerating system. The data were fitted to the Michaelis-Menten equation. PFP aqueous concentrations were calculated using 1.153 nM (in the buffer) per ppm in the gas phase (see text for details).

Figure 4. Representative $V/[S]$ plots for the formation of TFPG in live and heat-inactivated male rat liver microsomes in the presence of 10 mM GSH. The linear curve-fit was forced to intercept with the origin. PFP aqueous concentrations were calculated using 1.153 nM (in the buffer) per ppm in the gas phase (see text for details).

Figure 5. Semilogarithmic plot of PFPG level in 0.1 M potassium phosphate buffer at pH 7.4 and 37°C as a function of time.
Table 1. Maximum velocity ($V_{\text{max}}$), Michaelis-Menten constant ($K_m$), and intrinsic clearance ($V_{\text{max}}/K_m$) values of PFP metabolism in male and female rat, mouse, dog, and human microsomes via TFPA formation pathway.

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<tr>
<th>Parameter</th>
<th>Rat</th>
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</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>327.0 ± 50.4</td>
<td>432.9 ± 122.1</td>
<td>523.4 ± 112.5</td>
<td>633.8 ± 111.1</td>
</tr>
<tr>
<td>($\text{pmol/min/mg}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$</td>
<td>1.77 ± 0.09</td>
<td>1.33 ± 0.20</td>
<td>1.89 ± 0.23</td>
<td>1.36 ± 0.14</td>
</tr>
<tr>
<td>($\mu\text{M}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}/K_m$</td>
<td>186.0 ± 35.8</td>
<td>320.6 ± 52.2</td>
<td>274.3 ± 29.3</td>
<td>466.3 ± 81.1</td>
</tr>
<tr>
<td>($\mu\text{L/min/mg}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Values were obtained from fitting the $V/[S]$ curves to the Michaelis-Menten equation and averaged from at least three individual enzymatic reactions. Enzymatic reaction was conducted with microsomal protein concentration at 0.5 mg/mL and incubated for 20 min in 0.1 M potassium phosphate buffer (pH 7.4) in the presence of the NADPH-regenerating system at 37°C.
Table 2. Intrinsic clearance ($V_{\text{max}}/K_{m}$) values of PFP metabolism in male and female rat, mouse, dog, and human microsomes and cytosol via GSH $S$-conjugation pathway.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Rat</th>
<th>Mouse</th>
<th>Dog</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>TFPG, microsome</td>
<td>32.47 ± 4.49</td>
<td>50.45 ± 4.33</td>
<td>13.64 ± 0.67</td>
<td>5.53 ± 1.29</td>
</tr>
<tr>
<td>TFPG, cytosol</td>
<td>1.38 ± 0.48</td>
<td>1.98 ± 0.72</td>
<td>2.85 ± 0.86</td>
<td>1.64 ± 0.47</td>
</tr>
<tr>
<td>PFPG, microsome</td>
<td>3.34 ± 0.56</td>
<td>3.48 ± 0.52</td>
<td>2.24 ± 0.21</td>
<td>0.54 ± 0.71</td>
</tr>
<tr>
<td>PFPG, cytosol</td>
<td>4.72 ± 0.74</td>
<td>3.61 ± 0.66</td>
<td>5.32 ± 0.73</td>
<td>5.32 ± 0.64</td>
</tr>
</tbody>
</table>

$V_{\text{max}}/K_{m}$ $^a$ ($\mu$L/h/mg)

$^a$ Intrinsic clearance as a result of enzymatic reaction. Clearance rates from non-enzymatic, spontaneous reactions were obtained from heat-inactivated microsomes or cytosol and were subtracted from the rates obtained in live microsomes or cytosol. Values were averaged from the $V/[S]$ ratios at one to four different PFP concentrations that are low relative to the $K_{m}$ and were from at least three individual enzymatic reactions. Enzymatic reaction was conducted with microsomal and cytosol protein concentrations at 0.2 and 0.5 mg/mL, respectively, and incubated for 60 min in 0.1 M potassium phosphate buffer (pH 7.4) in the presence of 10 mM GSH at 37°C.

$^b$ PFPG was quantified by LC/MS method using TFPG as the analytical standard.
Table 3. Enzyme kinetic parameters for PFP metabolism in freshly isolated male rat hepatocytes via the formation of TFPA, TFPG, and PFPG pathways.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TFPA</th>
<th>TFPG</th>
<th>PFPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (pmol/min/10^6 cells)</td>
<td>65.16 ± 5.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsome equivalent $V_{max}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pmol/min/mg) $^d$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>0.62 ± 0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}/K_m$</td>
<td>165.79 ± 128.52</td>
<td>15.12 ± 0.82 $^c$</td>
<td>3.08 ± 0.47 $^c$</td>
</tr>
<tr>
<td>(µL/min/10^6 cell)</td>
<td>(µL/h/10^6 cell)</td>
<td>(µL/h/10^6 cell)</td>
<td></td>
</tr>
<tr>
<td>Microsome equivalent</td>
<td>497.37</td>
<td>45.36</td>
<td>9.24</td>
</tr>
<tr>
<td>$V_{max}/K_m$ $^d$</td>
<td>(µL/min/mg)</td>
<td>(µL/h/mg)</td>
<td>(µL/h/mg)</td>
</tr>
</tbody>
</table>

$^a$ Values were obtained from four individual reactions and two hepatocyte isolations with a total of 4 animals.

$^b$ PFPG was quantified by LC/MS method using TFPG as the analytical standard.

$^c$ Values include contributions from spontaneous, non-enzymatic reactions.

$^d$ For easy comparison with Table 1 and 2, unit was converted assuming 1 mg of microsomal proteins is equivalent to 3 million hepatocytes (Houston, 1994). Only the mean was used for this conversion.
Figure 1

DMD #38299

P450-catalyzed oxidative dehalogenation

\[
\begin{align*}
\text{TPA} & : & \text{H} & \text{C} & \text{F}_3 \\
\text{TFPA} & : & \text{H} & \text{C} & \text{F}_3 \\
\text{PFP} & : & & & \\
\text{TFPG} & : & \text{H} & \text{S} & \text{C} & \text{F}_3 \\
\text{PFPG} & : & \text{H} & \text{S} & \text{C} & \text{F}_3 \\
\end{align*}
\]

GST-catalyzed glutathione S-conjugation
Figure 5

PFPG Peak Intensity (Arbitrary Unit)

Time (hour)