Biological Cleavage of the C–P Bond in Perfluoroalkyl Phosphinic Acids in Male Sprague-Dawley Rats and the Formation of Persistent and Reactive Metabolites

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BACKGROUND: Perfluoroalkyl phosphinic acids (PFPiAs) have been detected in humans, wildlife, and various environmental matrices. These compounds have been used with perfluoroalkyl phosphonic acids (PFPA s) as surfactants in consumer products and as nonfoaming additives in pesticide formulations. Unlike the structurally related perfluoroalkyl sulfonic and carboxylic acids, little is known about the biological fate of PFPiAs.

OBJECTIVES: We determined the biotransformation products of PFPiAs and some pharmacokinetic parameters in a rat model.

METHODS: Male Sprague-Dawley rats received an oral gavage dose of either C6/C8PFPiA, C6/C8PFPA, or C8PFPA. Blood was sampled over time, and livers were harvested upon sacrifice. Analytes were quantified using ultra-high-performance liquid chromatography–tandem mass spectrometry or gas chromatography–mass spectrometry.

RESULTS: PFPiAs were metabolized to the corresponding PFPA s and 1H-perfluoroalkanes (1H-PFAs), with 70% and 75% biotransformation 2 wk after a single bolus dose for C6/C8PFPiA and C6/C8PFPA, respectively. This is the first reported cleavage of a C-P bond in mammals, and the first attempt, with a single-dose exposure, to characterize the degradation of any perfluoroalkyl acid. Elimination half-lives were 1.9 ± 0.5 and 2.8 ± 0.8 days for C6/C8PFPA and C6/C8PFPiA, respectively, and 0.95 ± 0.17 days for C8PFPA. Although elimination half-lives were not determined for 1H-PFAs, concentrations were higher than the corresponding PFPA s 48 h after rats were dosed with PFPiAs, suggestive of slower elimination.

CONCLUSIONS: PFPiAs were metabolized in Sprague-Dawley rats to form persistent PFPA s as well as 1H-PFAs, which contain a labile hydrogen that may undergo further metabolism. These results in rats produced preliminary findings of the pharmacokinetics and metabolism of PFPA s, which should be further investigated in humans. If there is a parallel between the disposition of these chemicals in humans and rats, then humans with detectable amounts of PFPiAs in their blood may be undergoing continuous exposure.

Introduction

Perfluoroalkyl acids (PFAAs) are ubiquitous environmental contaminants that arise from direct usage as surfactants and as the ultimate breakdown products of many other fluorinated chemicals (Butt et al. 2014; D’eon and Mabury 2011; Young and Mabury 2010). Strong carbon–fluorine bonds make perfluoroalkyl chains resistant to biological degradation as well as stable in wide temperature, pH, and pressure ranges (Kissa 2001). The persistence, toxicity, and bioaccumulation potential of certain perfluoroalkyl chain lengths of perfluoroalkyl carboxylic and sulfonic acids (PFCAs and PFSA s) have resulted in numerous phase-outs and bans worldwide (3M 2000; U.S. EPA 2006). Although humans are exposed to an increasing amount of unknown organofluorine-containing molecules (Yeung and Mabury 2016), most pharmacokinetic research has focused on PFCAs and PFSA s.

Although they are structurally similar to PFCAs and PFSA s, there is a paucity of literature on perfluoroalkyl phosphonic and phosphinic acids (PFPAs and PFPiAs, respectively), which have the general structure of [F(CF2)2]2[P(O2)3] and F(CF2)2PO3−, respectively (Figure 1). PFPiAs and PFPA s with chain lengths of x = 6, 8, 10, 12 and y = 6, 8 (x + y ≤ 18 for PFPiAs) have been identified together in commercial mixtures for use as wetting agents in consumer products and have also been used as anti-foaming additives to pesticide formulations (D’eon et al. 2009; Wang et al. 2016). Their current usage patterns are not fully understood, and they have often been incorrectly grouped together with mono- and di-perfluoroalkyl phosphate esters in regulatory documents. The first environmental observation of any PFPiA or PFPA was when C6, C8, and C10 PFPA s were detected in Canadian surface water and wastewater treatment plant effluent samples collected from 2004–2007 (D’eon et al. 2009). PFPA s were not targeted analytes in that research.

To our knowledge, there have only been three studies that reported screening for PFPiAs and PFPA s in humans. Although many monitoring programs measure other per- and polyfluorinated substances (PFASs) in the environment, PFPiAs and PFPA s are generally not included as analytes. PFPA s are more challenging to analyze owing to low analytical sensitivity caused by their minus-two charge state. Lee and Mabury (2011) searched for PFPiAs and PFPA s in human sera collected from U.S. residents in 2009; they reported PFPiAs in human sera for the first time but did not detect any PFPA s. The most commonly detected congeners were C6/C8PFPiA and C8/C8PFPA, which were found in >50% of samples at concentrations from 4–38 pg/mL (Lee and Mabury 2011). Another study reported PFPiAs, and for the first time PFPA s, in human plasma collected as early as 1985 from two German cities (Yeung and Mabury 2016). The authors used an instrument with lower detection limits than that used by Lee and Mabury (2011), which may have allowed them to detect PFPA s. The reported instrumental limit of quantification for all PFPiAs and PFPA s by Yeung and Mabury (2016) was 50 fg on-column in whole blood, compared with 0.5–9.0 pg on-column by Lee and Mabury (2011). PFPiA detections in German plasma samples were less frequent than in the American sera, but the most common congeners were also C6/C8PFPiA and C8/C8PFPA. German plasma also contained C6 and C8 PFPA s, with all analytes ranging from <10–50 ng/L. Neither PFPiAs nor PFPA s were detected in whole blood collected in 2004 from seven Chinese cities (Yeung et al. 2007).
PFPAs have been used for patterns of these compounds worldwide and show that PFPiAs and acid. The structures are depicted with charge states at pH 7. amu, atomic mass units; PFPA, perfluoroalkyl phosphonic acid; PFPiA, perfluoroalkyl phosphinic acid.

Although there have only been a handful of measurements in humans, there have been additional reports of other environmental PFPiA and PFPA contaminations. Humans may be exposed through household dust (De Silva et al. 2012) and tap water, where only PFPAs were measured (Llorca et al. 2012). Interestingly, PFPAs have recently been detected in 100% of serum samples from dolphins, cormorants, and pike in North America, with total PFPiA levels reported at 1.87 ± 2.17 ng/g wet weight on average (De Silva et al. 2016). Another recent paper reported PFPA contamination in sediment from Lake Ontario and two small lakes in the province of Ontario (Guo et al. 2016). The structure of PFPAs suggests that they would be persistent against biological transformations because of the perfluoroalkyl chains and the stable carbon–phosphorus bonds that link the hydrophobic and lipophobic perfluoroalkyl groups of the surfactant to the polar head group. A study in juvenile rainbow trout reported the intriguing in vivo formation of PFPAAs from PFPiAs, although the rest of the molecule was not accounted for (Lee et al. 2012). Despite their being detected in humans and wildlife, there is no published information on the toxicology or reactivity of these compounds in mammals.

To better understand the fate of PFPiAs in mammals, rats were dosed with either C6/C8 PFPiA, C8/C8 PFPiA, or C8 PFPA. To determine if PFPiAs degrade to PFPAAs in rats and to establish some preliminary pharmacokinetic parameters, rats received a 50-μg/kg single bolus dose via oral gavage (n = 3 per treatment). Blood was sampled over time to determine the pharmacokinetics of the individual molecules, and livers were harvested to assess distribution. We hypothesized that 1H-perfluoroalkanes (1H-PFAs) would form as the presumed carbanion leaving group of the PFPiA molecules. To test that hypothesis, a group of rats was redosed with the same molecules at 2 mg/kg (n = 3 per treatment and one control). A higher concentration dosage was required because of the volatility and analytical challenges involved in measuring 1H-PFAs.

Methods

Chemicals

All PFPiA, PFPA, and PFCA standards were obtained from Wellington Laboratories. 1H-PFAs were purchased from Synquest Laboratories. For complete details of chemicals used, see Supplemental Material (“Chemicals”).

Animal Treatment and Chemical Administration

All work was performed at the University of Toronto’s Division of Comparative Medicine under an animal use protocol approved by the university’s Animal Care Committee in compliance with the guidelines of the Canadian Council on Animal Care (www.cccac.ca). All animals were treated humanely with regard to alleviation of suffering. Thirty 7-wk-old male Sprague-Dawley rats were obtained from Charles River Laboratories. The animals were housed in triplicate and were exposed to a 12-h light-dark cycle, with food and water available ad libitum. Rats were allowed to adjust to their new environment for 17 d before the first blood collection. Whole blood was collected throughout the experiment into Sarstedt Microvette® CB 300 lithium heparin vials from the tail vein when a single sample was required and from the saphenous veins when multiple samples were required in a day. Blood was collected 3 d before dosing to assess any preexisting contamination. At the time of dosing, rats were 10 wk old and weighed 372 ± 10 g (range: 350–389 g). Three groups of 9 rats were administered either C6/C8 PFPiA, C8/C8 PFPiA, or C8 PFPA at 50 μg/kg via oral gavage at 4 mL/kg without prior fasting. This concentration corresponds to 62 mmol/kg C6/C8 PFPiA, 55 mmol/kg C8/C8 PFPiA, or 100 mmol/kg C8 PFPA. Dosing solutions were prepared in a vehicle of 50:50 propylene glycol:water with 0.1% soy lecithin as an emulsifier to assist solubilization (D’eon and Mabury 2010). Three control rats were dosed with the vehicle only. Approximately 150 μL of blood was sampled 0.5, 2, 4, 8 h, 1 d, 3 d, 4 d, 7 d, 10 d, and 14 d postdose. Three rats from each treatment group and one control rat were sacrificed after 3 and 7 d. Blood from time points 0.5 h to 3 d had n = 9 per treatment; blood from days 4 and 7 had n = 6 per treatment; and blood from days 10 and 14 had n = 3 per treatment. Terminal procedures were performed under 2000 mg/kg urethane anesthesia. Urethane was chosen instead of more commonly used halogenated anesthetics to prevent analytical signal overlap, which can occur in our methods. Approximately 5 mL of whole blood was collected via cardiac puncture into BD Vacutainer® tubes with lithium heparin as an anticoagulant. The animals were sacrificed by cervical dislocation, and liver samples were collected and stored in 50-mL Falcon tubes at −20°C until extraction. All blood was stored at 4°C.

To search for potential 1H-PFA metabolites of PFPAAs and PFPiAs, the 10 rats sampled up to 14 d were redosed at a higher concentration (2 mg/kg) of the same chemical that they were first administered (n = 3 per treatment, plus one control). This concentration corresponds to 2.5 mol/kg C6/C8 PFPiA, 2.2 mol/kg C8/C8 PFPiA, or 4.0 mol/kg C8 PFPA. The rats, then 528 ± 34 g (range: 488–570 g), were sampled for blood after 0.5 h, 2 h, 4 h, 8 h, and 1 d. Rats were sacrificed 48 h after dosing, and blood and liver samples were collected.

Figure 1. Structures of the PFPiAs and PFPAAs investigated in this study. Molar masses are listed as the fully protonated forms of the molecules, but the structures are depicted with charge states at pH 7. amu, atomic mass units; PFPA, perfluoroalkyl phosphonic acid; PFPiA, perfluoroalkyl phosphinic acid.
Extraction Procedures
Concentrations were measured in whole blood instead of plasma or serum because of evidence showing that PFPA s may enter cellular components (D’eon and Mabury 2010). To extract 100 μL of whole blood, 300 μL of ice-cold acetonitrile (ACN) was added, and the sample was vortexed and then centrifuged at 14,000 × g for 10 min at 4°C. The supernatant was transferred to a clean vial for ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analysis. Livers were homogenized using a Tissue Tearor (Biospec Products) with 1 g liver (wet weight) and 0.2 mL 1% potassium chloride solution; the homogenate was then extracted with 2.5 mL ACN acidified to pH 3 with formic acid. After centrifugation for 10 min at 4,000 × g, the supernatant was transferred to a clean tube, and the extraction was repeated twice. Combined extracts were evaporated to dryness and reconstituted in 1 mL methanol for analysis with UPLC-MS/MS.

Instrumental Analysis
UPLC-MS/MS. Analysis of PFPA s, PFPA s and PFCA s was performed using a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer operating in negative ion mode for electrospray ionization. A Waters Acuity UPLC BEH C18 column (2.1 mm × 75 mm, 1.7 μm) was heated to 60°C with a flow rate of 0.5 mL/min. Solvent composition began at 95% A [0.1% ammonium hydroxide (NH₄OH)] and 5% B (methanol) and was held at those conditions until 0.5 min, when it was ramped to 30% B. Between 1.0 and 5.5 min, B was increased to 95% and was held there until 6.0 min. At 6.1 min, the composition was returned to 5% B, for a total run time of 8 min. Sample injection volume was 2 μL. Mass spectrometry parameters used for all analytes have been published previously (Yeung and Mabury 2016).

Because no mass-labeled standards were available for PFPA s or PFPA s, matrix-matched calibration curves were prepared using control rat samples. For the high-concentration experiment, samples had to be diluted 100× in methanol to analyze dosed molecules (not metabolites); therefore, calibration standards were prepared in methanol.

GC-MS with solid phase microextraction. To probe for the formation of volatile metabolites, approximately 5 mL of blood collected from the cardiac puncture of the high-dose experiment was analyzed for 1H-PFA s. Analytes were extracted using headspace solid phase microextraction (SPME) directly into sealed Vacutainers®, which had been stored at 4°C and never opened to ensure that the volatile molecules would not escape. Blood was heated at 30°C for 20 min to develop an equilibrium between the liquid and gas phases, and the SPME fiber (100 μm polydimethylsiloxane (PDMS) on fused silica; Supelco) was exposed to the headspace for 5 min before GC-MS analysis. To quantify the 1H-perfluorohexane and 1H-perfluorooctane in the blood samples, matrix-matched calibration curves were prepared specifically for each blood sample at the same volume (3.4–6.8 mL). SPME relies on liquid/gas phase partitioning; thus, the volume must be consistent when comparing samples to standards. Owing to the low water solubility of 1H-PFA s, ACN was used as a cosolvent when preparing standards, with a final volume of <3% in the spiked blood.

Samples were analyzed using an Agilent 7890A gas chromatograph with a 5975C MSD mass spectrometer operating in negative chemical ionization mode with methane as a reagent gas. An Agilent GS-GasPro column was used (30 m length, inner diameter 0.32 mm) with helium as a carrier gas (1.8 mL/min). The GC inlet was in splitless mode and was heated to 200°C to desorb the analytes from the SPME fiber. The GC temperature profile ramped from 140°C to 250°C. The instrument was operated in selected ion monitoring (SIM) mode. For both 1H-perfluorohexane and 1H-perfluorooctane, the [M-HF]⁻ peaks of m/z 300 and 400, respectively, were used for quantification. The [M-H]⁻ and [M-HF-CF₂]⁻ peaks were used as confirmation peaks. The retention times were 5.3 min for 1H-perfluorohexane and 8.9 min for 1H-perfluorooctane. Samples were analyzed in triplicate with analytical standard deviations of <3% for all samples.

Quality Control
Limits of detection and quantification. Limits of detection and quantitation (LODs and LOQs, respectively) were determined using blank blood and liver matrix extracts that were spiked with a mixture of analytes. The limits were empirically determined using concentrations that produced signal-to-noise ratios of 3 for LOD and 10 for LOQ. Values determined for analysis using UPLC-MS/MS are reported as picomoles/gram blood as follows: C₆/C₆PFPA, LOD: 0.0050, LOQ: 0.025; C₈/C₈PFPA, LOD: 0.0020, LOQ: 0.022; C₁₀PFPA, LOD: 0.050, LOQ: 0.10; C₁₂ PFPA, LOD: 0.080, LOQ: 0.40; perfluorohexanoic acid PFHxA, LOD: 0.64, LOQ: 1.3; perfluorooctanoic acid (PFHxA), LOD: 0.55, LOQ: 1.1; perfluorooctanoic acid (PFOA), LOD: 0.77, LOQ: 0.97; perfluorononanoic acid (PFNA), LOD: 0.43, LOQ: 0.86.

In liver extracts, the LODs and LOQs are reported as picomoles/g gram liver as follows: C₆/C₆PFPA, LOD: 0.0010, LOQ: 0.0050; C₈/C₈PFPA, LOD: 0.0010, LOQ: 0.004; C₁₀PFPA, LOD: 0.015, LOQ: 0.050; C₁₂ PFPA, LOD: 0.022, LOQ: 0.072; PFHxA, LOD: 0.19, LOQ: 0.64; PFHpA, LOD: 0.16, LOQ: 0.55; PFODA, LOD: 0.034, LOQ: 0.116; PFNA, LOD: 0.0080, LOQ: 0.028.

For analytes measured using SPME and GC-MS, LODs and LOQs were 8.8 and 29 pmol/g blood, respectively, for 1H-perfluorohexane, and 1.2 and 3.8 pmol/g blood for 1H-perfluorooctane.

Blanks. Blood collected three days before the first oral gavage was free of PFPA s, PFPA s, and all PFCA s except for PFOA. Throughout the study, PFOA levels were 0.029 ± 0.025 ng/g [standard deviation (SD); n = 17] in the control blood samples. Method LODs and LOQs for PFOA were calculated as the blank levels plus 3 or 10 times the standard deviation to determine the LOD and LOQ of PFOA in blood, respectively, resulting in an LOD of 0.11 ng/g and an LOQ of 0.28 ng/g for PFOA. All blood from control rats was free of PFPA and PFPA contamination throughout the study, as were the livers from the control rats sacrificed 3 and 7 d after rats were dosed at 50 μg/kg. The control rat sacrificed 48 h after rats were dosed at 2 mg/kg had some contamination in the liver, which may have occurred from cross-contamination during the dissection or from general background contamination depending on the analyte. Levels were 0.088 ng/g PFOA, 0.089 ng/g PFNA, 1.1 ng/g C₈PFPA, 12 ng/g C₁₀PFPA, 2.4 ng/g C₁₂/C₁₀PFPA, and 1.3 ng/g C₁₂/C₁₀PFPA. All of these concentrations were at least an order of magnitude lower than the levels in the other rats; thus, the concentrations were not corrected. Analytical method blanks were extracted alongside all samples and were always clean.

Extraction recovery. To determine the extraction recovery from whole blood, 40-ng/mL spike solutions of bovine blood (purchased from BioChemed) were prepared in triplicate. Recovery was 91 ± 5% (SD) for C₆/C₆PFPA, 90 ± 8% for C₈/C₈PFPA, 63 ± 4% for C₁₀ PFPA, and 67 ± 3% for C₁₂ PFPA. To determine the extraction recovery from liver, 1 ng of each analyte was spiked into liver homogenate in triplicate. Recovery was 90 ± 3% for C₆/C₆PFPA, 78 ± 4% for C₈/C₈PFPA, 65 ± 3% for C₁₀ PFPA, and 52 ± 4% for C₁₂ PFPA. Recovery for PFCA s ranged from 64–81%. Concentrations were not recovery corrected.


**Stability tests.** To confirm that the formation of PFPAAs and 1H-perfluoroalkanes from PFPIAs was a unique biological process, stability tests were performed using deionized water and bovine blood. Solutions of 40 ng/mL C₆/C₈ PFPIA were prepared in water and blood and were transferred to small vials to be extracted and analyzed over time. Concentrations of C₆/C₈ PFPIA were stable over one week, and there was no formation of PFPAAs observed. An analogous test was performed in aqueous sodium hydroxide at pH ~ 13. Here, we saw the formation of C₆ PFPA and C₈ PFPA over time, similar to what was observed for the alkaline hydrolysis of C₆/C₈ PFPIA (Emeléus and Smith 1959).

**Statistical Analysis**

Data analyses were performed using OriginPro 2017 (Originlab Corporation). For the purpose of calculating means, all values below the limit of detection were treated as one half of the LOD. Absorption and elimination half-lives were calculated using a one-compartment model to obtain clear preliminary estimates of pharmacokinetic values. Assuming first-order absorption and elimination, the following applies for the amount of contaminant in the blood:

\[
\frac{dA_B}{dt} = k_{abs}A_{GI} - k_{elim}A_B,
\]

where \( k_{abs} \) is the first order absorption rate from the gastrointestinal tract \((GI)\), and \( k_{elim} \) is the first order elimination rate from the blood \((B)\).

Both the absorption and excretion half-lives \((t_{1/2})\) are calculated from the rate constants:

\[
t_{1/2} = \frac{-0.693}{k}.
\]

A plot of ln \( C_B \) versus time shows a linear relationship when \( k_{abs} = 0 \), and the slope of that section is \(-k_{elim}\). To calculate \( t_{1/2, abs} \), concentrations during the absorption phase were back-extrapolated to obtain a concentration defined as \( C_B' \); \( C_B' - C_B \) represents the amount absorbed from the GI tract after exposure. A plot of ln \( (C_B' - C_B) \) versus time results in the slope of \(-k_{abs}\), which allows the calculation of \( t_{1/2, abs} \) to be calculated. The errors for both half-lives were calculated using the errors from the slope of the line of best fit.

The extent of biotransformation of PFPIAs was calculated using the integration function to obtain the areas under the curve (AUC) of PFPIAs and PFPAAs in the plots of molar concentration versus time (Figure 2; Table S2). Using the AUC allowed us to account for the extent of excretion that occurred over time. Because one PFPIA molecule yields one PFPA molecule, the percent biotransformation was calculated after 14 d using the following equation:

\[
\%\,\text{biotransformation}_{14} = \frac{\sum \text{AUC}(\text{mol PFPA})_{14}}{\text{AUC}(\text{mol PFPIA})_{14}} \times 100. \quad [3]
\]

Comparisons between concentration levels of different analytes and biological compartments (Figures 3 and 4) did not have enough samples \((n = 3)\) for true statistical power; therefore, observations are discussed in “Results” and “Discussion.”

**Results**

In order to begin to assess the potential for human exposure, our aim was to understand the elimination kinetics, bio-

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**Table 1.** Pharmacokinetic parameters in Sprague-Dawley rats after oral gavage at 50 µg/kg.

| Dosed molecule | \( t_{1/2} \), Absorption, h\( ^a \) | \( t_{1/2} \), Elimination, d\( ^a \) | \( T_{max} \), h | \( C_{max} \), ng/g (mean ± SE) | Percent biotransformation, \( t = 14 \) d\( ^a \) |
|---------------|------------------|------------------|--------|-------------------|------------------|
| C₆/C₈ PFPIA   | 2.7 ± 0.5        | 1.9 ± 0.5        | 8      | 63 ± 6            | 70%              |
| C₆/C₈ PFPA    | 1.3 ± 0.4        | 2.8 ± 0.8        | 4      | 17 ± 4            | 75%              |
| C₈ PFPA       | 2.1 ± 1.2        | 0.93 ± 0.17      | 8      | 24 ± 8            | —                |

Note: —, not applicable; \( C_{max} \), maximum concentration; PFPA, perfluoroalkyl phosphonic acid; PFPIA, perfluoroalkyl phosphinic acid; \( t_{1/2} \), absorption half-life in blood; \( t_{1/2} \), elimination half-life in blood; \( T_{max} \), time of maximum concentration.

\( ^a \)Percent biotransformation was approximated for PFPAAs using PFPA metabolite concentrations.
transformation, and distribution of PFPiAs and their metabolites in rats as a mammalian model.

**Pharmacokinetics**

After the initial oral gavage, blood concentrations of PFPiAs and PFPAAs increased, corresponding to absorption from the gastrointestinal tract into the bloodstream, and then decreased as elimination occurred (Figure 2; Table S1). Whole-blood concentrations were obtained four times within the first day at 0.5, 2, 4, and 8 h postdose, with the maximum concentrations observed at 8 h for C8/C8 PFPiA and C8 PFPA and at 4 h for C6/C8 PFPiA (Table 1). The absorption half-life of C8/C8 PFPiA was the fastest, with a value of 1.3 ± 0.4 h, followed by C8 PFPA and C6/C8 PFPiA, with values of 2.1 ± 1.2 h and 2.7 ± 0.5 h, respectively. The greatest variability in concentrations between the nine replicates was during the absorption phase. Of the three molecules dosed, C8/C8 PFPiA had the highest measured concentration in blood (63 ± 6 ng/g); this value was more than twice the maximum concentration of C8 PFPA and more than three times the maximum concentration of C8/C8 PFPiA. Although the large molecular mass of C8/C8 PFPiA [902 atomic mass units (amu)] may suggest low bioavailability (Lipinski et al. 2001), our results are consistent with the bioavailability of PFPiAs observed in rats for the Masurf® FS-780 commercial mixture containing various congeners of PFPiAs and PFPAAs (D’eon and Mabury 2010). The authors suggested that the relatively high bioavailability may be a result of the large mass-to-volume ratio of the fluorine atoms on

![Figure 3](image3.png)

**Figure 3.** Concentrations in whole blood 48 h after dosage at 2 mg/kg. Of all measured analytes, the largest concentration was of the dosed molecules. Between the two classes of metabolites formed, there were greater concentrations of 1H-PFAs than PFPAAs. Each bar represents an average of 3 rats, with standard errors plotted as error bars. 1H-PFAs, 1H-perfluoroalkanes; PFPA, perfluoroalkyl phosphonic acid; PFPiA, perfluoroalkyl phosphinic acid.

![Figure 4](image4.png)

**Figure 4.** Whole blood and liver concentrations of PFPiAs and their PFPA metabolites. All liver-to-blood ratios (LBRs) were >1. At the 50 μg/kg dose, C8 PFPA LBRs were larger in rats dosed with PFPiAs than in rats dosed with C8 PFPA. At the 2 mg/kg dose, all C8 PFPA LBRs were the same regardless of whether the molecule was dosed or formed as a metabolite. Each bar represents an average of 3 rats, with standard errors plotted as error bars. PFPA, perfluoroalkyl phosphonic acid; PFPiA, perfluoroalkyl phosphinic acid.
the molecules, and they also suggested that the PFPIAs may be more bioavailable than PFPAIs owing to the difference in their physiological charge states (D’eon and Mabury 2010).

First-order elimination kinetics were determined in blood using data collected after the maximum concentration was reached, up to 14 d postdose. This is the first mammalian study of PFPIAs and PFPAIs; therefore, many unknown parameters remain, such as partitioning between compartments, including noncovalent interactions with proteins that have been observed with other PFAAs (Jones et al. 2003). Using a one-compartment model provides the first estimates of pharmacokinetic parameters. Correlation coefficients (r) ranged from 0.67 to 0.82 for elimination data and from 0.53 to 0.87 for absorption data. Elimination was most rapid for C8 PFPA, with a half-life of 0.95 ± 0.17 d, followed by C6/C8 PFPIA, with a half-life of 1.9 ± 0.5 d, and C8/C8 PFPIA, with a half-life of 2.8 ± 0.8 d (Table 1). Elimination processes for PFPIAs included both excretion and biotransformation. Throughout the 14 d after the single bolus dose, PFPAIs were formed in rats dosed with PFPAIs, and the concentrations were monitored over time (Figure 2; Table S1). This finding confirmed the preliminary observation of PFPA formation in juvenile rainbow trout dosed with PFPAIs (Lee et al. 2012). All samples were also analyzed for PFCAs as potential breakdown products of PFPAIs and PFPAIs. None of these compounds was above the limit of detection, except for PFOA, which could not be confirmed owing to low-level contamination. Therefore, there was no evidence of degradation for PFPAIs. C6 and C8 PFPAIs were formed in rats dosed with C6/C8 PFPIA, and only C8 PFPA was formed in rats dosed with C8/C8 PFPIA. Detection first occurred after 29 h, and after 14 d, the concentrations of the PFPA metabolites exceeded the concentration of the parent PFPAI molecules. After 14 d, the percent biotransformations of C6/C8 PFPIA and C8/C8 PFPIA to PFPAIs were approximately 70% and 75%, respectively, calculated using the AUC for PFPAI and PFPAI molar blood concentrations over time (Figure 2; Table S2). Biotransformation appeared to be the dominant elimination process of PFPIAs in rats, which corroborates the low proportion of PFPIAs found in urine and feces of rats dosed with the commercial mixture (D’eon and Mabury 2010).

The whole-blood elimination half-lives we report for C6/C8 PFPIA and C8/C8 PFPIA align with those reported by D’eon and Mabury (2010) after intraperitoneal injection of the Masurf® FS-780 commercial mixture that contained PFPIAs and PFPAIs. However, the elimination half-life of C8 PFPA that we report is 0.95 ± 0.17 d, compared with the 1.6 ± 0.1 d reported for male rats by D’eon and Mabury (2010). The simultaneous elimination of PFPAIs and formation of PFPAIs from PFPIAs in the mixture masked the absolute elimination of the dosed PFPA molecules, resulting in an apparent elimination rate two times slower than that reported herein. Thus, PFPA elimination in rats is faster than originally reported.

**Formation of 1H-PFAs**

To completely characterize the biotransformation of PFPIAs, the entire parent molecule must be accounted for in the products. We hypothesized that cleavage of the C–P bond would yield the corresponding PFPAIs and 1H-PFAs, presumably via protonation of a carbanion leaving group. When rats were dosed at 2 mg/kg, 1H-perfluorohexane (1H-PFHx) was detected in the blood of rats dosed with C8/C8 PFPIA, and 1H-perfluoroctane (1H-PFO) was detected in the blood of rats dosed with either C6/C8 PFPIA or C8/C8 PFPIA. 1H-Perfluoroctane was not detected in the blood of rats dosed with C8 PFPA, meaning that PFPAIs themselves do not undergo a similar hydrolysis of the C–P bond in vivo. Blood concentrations from the 2 mg/kg dose are reported in Figure S1 and Tables S3 and S4.

Upon sacrifice, the blood from rats dosed with either of the two PFPIA molecules contained more 1H-PFA metabolites than PFPA metabolites (Figure 3). Assuming one PFPIA equivalent is broken down to one PFPA equivalent and one 1H-PFA equivalent, their concentrations would be equal in the blood if they had the same behavior in the body. Rats dosed with C6/C8 PFPIA contained 22 times (±5 SD, n = 3) as much 1H-perfluorohexane as C8 PFPA and 23 ± 9 times as much 1H-PFHx as C8 PFPA. Rats dosed with C8/C8 PFPIA contained 7.2 ± 1.3 times as much 1H-PFO as C8 PFPA (Figure 3). A sample size of three rats per treatment was insufficient for meaningful statistical comparisons; therefore, this observation should be further investigated. Either PFPAIs were being excreted faster, or they were partitioning out of the blood into other biological compartments. The blood elimination half-life of C8 PFPA (determined to be <1 d; Table 1) aligns with the faster disappearance of PFPAIs compared with 1H-PFAs in blood. Unlike PFPAIs, 1H-PFAs do not contain a polar functional head group and are sparingly soluble in water, which would limit renal excretion and may lead to bioaccumulation. It is important to note that the 1H-PFAs were measured in blood but may be even more concentrated in less-aqueous biological compartments. We chose to measure 1H-PFAs in blood because the use of headspace SMPE allowed us to preclude analyte losses through volatilization during sample preparation. Subsequent studies investigating the disposition of 1H-PFAs will require robust methods in a broader range of sample matrices. At the present time, there is no literature on the elimination route and kinetics of 1H-PFAs.

**Partitioning into Liver**

The literature indicates that PFPAIs and PFPIAs predominantly partition into proteinaceous parts of rats (D’eon and Mabury 2010) and fish (Lee et al. 2012), with the highest concentrations found in the liver. Understanding the partitioning of both parent and metabolite molecules in the body can provide insight into where metabolism is occurring. In our work, all analytes had liver-to-blood ratios (LBRs) > 1 (Figure 4; Tables S5 and S6). In rats dosed at 50 µg/kg, the highest LBR was 47 ± 12 (SD; n = 3) for C8/C8 PFPIA after 3 d. This value decreased to 4.2 ± 0.5 after 7 d. C8 PFPA measured in the rats dosed with that molecule only had a lower LBR than rats dosed with either C6/C8 PFPIA or C8/C8 PFPIA, which formed C8 PFPA as a metabolite (Figure 4; Table S6). This observation may give insight into the liver’s role in the metabolism: If the PFPA metabolites are being formed in the liver, their concentration will be the highest there before being dispersed throughout the bloodstream. Lee et al. (2012) reported greater LBRs for PFPIAs than for PFPAIs in juvenile rainbow trout dosed with those molecules. It is possible that the higher concentration of PFPIAs in the liver allows for higher concentrations of PFPAIs to be formed as metabolites rather than if the PFPAIs were simply transferred from the blood to the liver. When rats were dosed at 2 mg/kg, the LBRs of C6/C8 PFPIA and C8/C8 PFPIA were lower after 2 d than they were after 3 d at the lower concentration dosage. Additionally, there was no difference in the LBRs of C8 PFPA based on whether it was dosed to the rats or formed as a metabolite in vivo. If metabolism is occurring in the liver, there may be a threshold that is being reached at this higher concentration.

**Discussion**

PFPIAs have been detected in humans, wildlife, and the environment, whereas PFPA contamination has been reported less frequently. Surprisingly, given the interesting chemistry surrounding
PFPiAs and PFPAs, few researchers have included them in their targeted analyze list. Additionally, PFPAs are relatively less sensitive than other PFAAs. The aim of this work was to determine if PFPiAs are metabolized to PFPAs in rats, similar to what was observed in juvenile rainbow trout (Lee et al. 2012). After confirming that observation, we then determined that the other half of the C8 PFPiA molecule was forming 1H-PFAs by detecting them in the blood of rats exposed to higher concentrations of PFPiAs. Rats exposed to C8 PFPiA did not form the corresponding 1H-PFA, confirming that PFPAs are persistent in a manner similar to all other PFAAs. 1H-PFAs contain a labile hydrogen atom that may undergo further biotransformation. We will discuss possible metabolic end points of 1H-PFAs as they relate to literature information on 1H-perfluoroethane (Harris et al. 1992) and 8:2 fluorotelomer alcohol (Martin et al. 2009; Rand and Mabury 2014).

### Cleavage of the C–P Bond

This work detected the first metabolism of any perfluoroalkyl acid (PFAA) identified in a mammalian species, where the C–P bond of PFPiAs was enzymatically cleaved in rats. However, we did not observe a similar cleavage of the C–P bond in PFPAs, which would result in 1H-PFAs and phosphate ions. The phosphorus atoms in PFPAs are relatively more electron-rich than the phosphorus atoms in PFPiAs, which may make them less susceptible to nucleophilic attack. PFPAs have one fewer electron-withdrawing perfluoroalkyl group than PFPiAs, and they also have an additional acidic group, which donates electrons to the phosphorus atom at biological pH. Although both molecules are anions, the single versus double charges of PFPiAs and PFPAs, respectively, may have significant impacts on their reactivity if it is enzymatically controlled.

To our knowledge, this is the first reported cleavage of the C–P bond in mammals. The only reported C–P enzymes are in bacteria such as Escherichia coli (McGrath et al. 2013), but PFPiAs could not undergo the same oxidative mechanisms owing to the location of the fluorine atoms on the molecules. One hypothesis is that enzyme promiscuity is occurring, wherein an enzyme catalyzes a reaction other than that for which it appears to be designed. An example of this phenomenon is paraoxon, the active metabolite of the pesticide parathion, which is metabolized by enzymes with native lactonase activity (Khersonsky and Tawfik 2005).

The observed liver-to-blood ratios suggest that biotransformation occurred in the liver. The continuous formation of PFPAs metabolites excludes the gut as a major location of metabolism. After a single bolus dose of either C8/C9 PFPiA or C8/C9 PFPAs, PFPA concentrations increased and then remained approximately stable throughout the 14-d experiment, even with the relatively fast elimination rate of PFPAs. For the asymmetric C8/C9 PFPiA, there was no apparent preference in the location of the C–P bond cleavage. Slightly higher levels of both C8 PFPA and 1H-PFHx were detected over C8 PFPA and 1H-PFO (Figures 2 and 3). If there had been a preference, we would have observed greater amounts of either C8 PFPA and 1H-PFO or C8 PFPA and 1H-PFHx.

### Implications for Human Exposure

The PFAAs with the greatest amount of research, regulatory, and public interest have half-lives on the order of years in humans. These molecules do not undergo any biological transformations; thus, the only mode of elimination is by excretion. Perfluorooctane sulfonic acid (PFOS) contains eight perfluorinated carbons and has a half-life of 5.4 y in humans, and PFOA contains seven perfluorinated carbons and has a half-life of <3.8 years (Lau 2012). Within the carboxylic and sulfonic acid subclasses, there is a general trend of half-lives increasing with increasing numbers of perfluorinated carbons in the molecule (Martin et al. 2003). However, there are large differences between toxicokinetics in humans and those in laboratory animals in addition to prominent sex differences within some species. Furthermore, we have shown that in addition to excretion as an elimination process, PFPiAs undergo metabolism to form PFPAAs and 1H-perfluorooalkanes in rats, which further complicates extrapolating kinetics to human exposure. The results presented here provide hypotheses about human exposure that should be further investigated.

Half-lives in rats are faster than in humans, and although it is difficult to compare across species, we can gain insight by relating PFPiA data to what is known about PFOS and PFOA. The half-lives of PFOS and PFOA in male rats are 43 d and <6 days, respectively (Lau 2012). Our work revealed half-lives of <3 days for C8/C9 PFPiA and C8/C9 PFPiA in male rats, meaning that these molecules are eliminated approximately twice as fast as PFOA, and much faster than PFOS, in rats. Therefore, we would expect the half-lives in humans to be shorter for PFPiAs than for PFOS and PFOA, even though PFPiAs have more perfluorinated carbons. This discrepancy is largely due to metabolism as an additional route of elimination for PFPiAs, compared with only excretion for PFOS and PFOA, suggesting that humans and animals with detectable amounts of PFPiAs may be undergoing continuous environmental exposure to these chemicals. The biological impact of PFPiAs may be potentially greater than that of other PFAAs at a similar exposure level because of the formation of two prominent metabolites, PFPAAs, which are persistent, and 1H-PFAs, which may undergo further metabolism. With no information on the toxicology of PFPAAs and their metabolites, it is impossible to predict the physiological effects that may be occurring. Elimination kinetics of PFPAAs and PFPAAs have been investigated in juvenile rainbow trout using neat PFPA or PFPA material separately (Lee et al. 2012) and using a commercial mixture of PFPAAs and PFPAAs in rats (D’eon and Mabury 2010) and zebrafish (Chen et al. 2016). The concern with using the commercial mixture is that the elimination rate of PFPAAs will appear to be slower because their formation as a metabolite of PFPAAs masks the excretion that occurs simultaneously. Within this small body of literature, there is a notable difference in elimination rates for PFPAAs in rats versus rainbow trout and zebrafish. Elimination half-lives of C8/C9 PFPA and C8/C9 PFPA in male fish are reported here as 2.2 ± 0.2 and 2.8 ± 0.5 days, respectively, and by D’eon and Mabury (2010) as 1.9 ± 0.5 and 2.8 ± 0.8 days, respectively. However, the half-lives in rainbow trout for C8/C9 PFPA and C8/C9 PFPA were 20.4 ± 4.9 and 52.7 ± 15.8 days, respectively (Lee et al. 2012). Chen et al. (2016) found similar values in zebrafish to those reported in rainbow trout. The only data on PFPA elimination that are not affected by the use of a commercial mixture are the half-life in male rats of 0.95 ± 0.17 days reported here and the half-life in rainbow trout of 4.4 ± 0.7 days reported by Lee et al. (2012). Overall, rats have better capabilities for eliminating PFPAAs and PFPAAs from their systems.

In this work, we chose to use male Sprague-Dawley rats because other PFAAs have much faster elimination in females than in males. Specifically, PFOA has a half-life of 2–4 h in female rats compared with 4–6 d for male rats (Lau 2012). To ensure that we could monitor metabolite formation, we chose male rats, in case the females eliminated the PFPAAs or their metabolites too quickly. This factor was particularly important because PFOA was a potential metabolite that we did look for but did not detect. The vast differences between species for PFPA elimination may be caused by the differences in metabolic capabilities in addition to differences in excretion. In light of these
differences and the potential for sex-based differences, more research is needed to assess the exposure to PFPiAs of mammalian and aquatic species, including low-dose chronic exposure studies. In a mouse study of continuous PFOA exposure, a one-compartment kinetic model provided a poor fit for pharmacokinetic data (Lou et al. 2009). At the present time, no PFPiA or PFPA studies have used more sophisticated pharmacokinetic modeling; therefore, this should be pursued in future studies. The observed species-based differences motivate the need for more human-focused research.

**Potential Biotransformation of 1H-PFAs**

To our knowledge, this is the first observation of the formation of 1H-PFAs *in vivo*. The shorter hydrofluorocarbon analog 1H-perfluoroethane (i.e., HFC-125) has been shown to be metabolized in rats very slowly following a similar oxidative biotransformation pathway to the anesthetic halothane (Harris et al. 1992). Briefly, 1H-perfluoroethane is oxidized to an alcohol by cytochrome P450s, mainly CYP 2E1 (Dekant 1996), and then forms an acyl fluoride intermediate that can either form trifluoroacetylated proteins or trifluoroacetic acid. Halothane hepatitis is a type of liver necrosis caused by an immune response to trifluoroacetylated hepatic proteins (Ray and Drummond 1991), but in the case of halothane, the metabolism rates are much greater owing to the acyl chloride intermediate (vs. acyl fluoride), leading to more trifluoroacetylated proteins than from 1H-perfluoroethane (Harris et al. 1992). Cytochrome P450s have been proposed to be the enzymes responsible for oxidizing long-chain fluorotelomer molecules. Specifically, CYP 2E1 was reported to oxidize 8:2 fluorotelomer alcohol in rat hepatocytes, which provides evidence that compounds containing longer perfluoralkyl chains can still undergo these oxidative reactions (Martin et al. 2009). The 8:2 fluorotelomer unsaturated aldehyde reacted to form both PFCAs and to covalently bind to proteins both *in vitro* (Rand and Mabury 2013) and *in vivo* (Rand and Mabury 2014). The longer-chained 1H-PFAs would likely have similarly slow reactivity to 1H-perfluoroethane to form PFCAs or other biological nucleophiles such as certain amino acids in proteins. However, if excretion is slow, bio-transformation and subsequent reactions may be an important elimination route. In our work, we could not definitively report the formation of PFOA as a metabolite of C6/C8 PFPiA or C8/C6 PFPA because of low-level background contamination. Future experiments will test this hypothesis using *in vitro* techniques to minimize PFOA contamination and to improve detection limits.

**Conclusion**

We have shown that PFPiAs are cleaved *in vivo* to form persistent PFPA and labile 1H-PFAs in rats. This is the first reported *in vivo* formation of 1H-PFAs from any perfluorinated molecule; this is also the first attempt, with a single-dose exposure, to characterize the transformation of a perfluoroalkyl acid. Intriguingly, there are no known mammalian enzymes that carry out the biological cleavage of C–P bonds in phosphinic acids. The strong electron-withdrawing effects of two perfluoralkyl chains apparently increase the lability of the C–P bonds in PFPiAs compared with PFPA, which do not undergo an analogous transformation. These preliminary results in male Sprague-Dawley rats must be investigated further to determine their relevance to human exposure. PFPiAs have been detected in humans and wildlife; therefore, they may be continuous exposure to these chemicals because these molecules are presumably being degraded and excreted over time. Given the reported environmental detections and the unique biotransformation presented here, more research is required to understand the toxicokinetics and metabolism of human PFPiA exposure, including further biotransformation of 1H-PFAs.

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