



## Metabolic products and pathways of fluorotelomer alcohols in isolated rat hepatocytes

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### Abstract

Fluorotelomer alcohols (FTOHs;  $\text{CF}_3(\text{CF}_2)_x\text{C}_2\text{H}_4\text{OH}$ ; where  $x = 3, 5, 7, 9$ ) are a novel class of polyfluorinated contaminants, recently detected in the North American atmosphere, that are possible precursors to the series of perfluoroalkyl carboxylates (PFCAs) in human blood. An *in vivo* rat study validated earlier independent work that poly- and per-fluoroalkyl carboxylates were metabolites of FTOHs, but our detection of several novel metabolites prompted us to examine their pathways in greater detail using isolated rat hepatocytes. Using 8:2 FTOH (i.e. where  $x = 7$ ) as a model compound, the metabolic products formed by isolated rat hepatocytes were identified, and three synthesized intermediates were incubated separately to elucidate the metabolic pathways. For 8:2 FTOH, a major fate was direct conjugation to form the *O*-glucuronide and *O*-sulfate. Using 2,4-dinitrophenylhydrazine (DNPH) trapping, the immediate oxidation product of 8:2 FTOH was identified as 8:2 fluorotelomer aldehyde (8:2 FTAL;  $\text{CF}_3(\text{CF}_2)_7\text{CH}_2\text{C}(\text{H})\text{O}$ ). 8:2 FTAL was transient and eliminated HF non-enzymatically to yield 8:2 fluorotelomer  $\alpha,\beta$ -unsaturated aldehyde (8:2 FTUAL;  $\text{CF}_3(\text{CF}_2)_6\text{CF}=\text{CHC}(\text{H})\text{O}$ ) which was also short-lived and reacted GSH and perhaps other endogenous nucleophiles. Four polyfluorinated acid intermediates were also detected, including 8:2 fluorotelomer carboxylate (8:2 FTCA;  $\text{CF}_3(\text{CF}_2)_7\text{CH}_2\text{C}(\text{O})\text{O}^-$ ), 8:2 fluorotelomer  $\alpha,\beta$ -unsaturated carboxylate (8:2 FTUCA;  $\text{CF}_3(\text{CF}_2)_6\text{CFCHC}(\text{O})\text{O}^-$ ), tetrahydroperfluorodecanoate ( $\text{CF}_3(\text{CF}_2)_6(\text{CH}_2)_2\text{CO}_2^-$ ), and dihydroperfluorodecenoate ( $\text{CF}_3(\text{CF}_2)_6\text{CH}=\text{CHCO}_2^-$ ). The pathways leading to 8:2 FTCA and FTUCA involve oxidation of 8:2 FTAL, however, the pathways leading to the latter two polyfluorinated acids remain inconclusive. The fate of the unsaturated metabolites, 8:2 FTUAL and FTUCA, included conjugation with GSH and dehydrofluorination to yield  $\alpha,\beta$ -unsaturated GSH conjugates, and GS-8:2 FTUAL which was subsequently reduced to the corresponding alcohol. Perfluorooctanoate (PFOA) and minor amounts of perfluorononanoate (PFNA) were confirmed as metabolites of 8:2 FTOH, and the respective roles of  $\beta$ - and  $\alpha$ -oxidation mechanisms are discussed. The analogous acids, aldehydes, and

**Abbreviations:** DHPFCA, dihydroperfluoroalkyl carboxylate; DNPH, 2,4-dinitrophenylhydrazine; FTAL, fluorotelomer aldehyde; FTCA, fluorotelomer carboxylate; FTOH, fluorotelomer alcohol; FTUAL, fluorotelomer  $\alpha,\beta$ -unsaturated aldehyde; FTUCA, fluorotelomer  $\alpha,\beta$ -unsaturated carboxylate; HNA, 4-hydroxynonenic acid; HNE, 4-hydroxynonenal; HPLC/MS/MS, high pressure liquid chromatography tandem mass spectrometry; PFCA, perfluoroalkyl carboxylate; PFNA, perfluorononanoate; PFOA, perfluorooctanoate; THPFCA, tetrahydroperfluoroalkyl carboxylate

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conjugated metabolites of 4:2, 6:2, and 10:2 FTOH (i.e. where  $x = 3, 5,$  and  $9,$  respectively) were also detected, and metabolite profiles among FTOHs generally differed only in the length of their perfluoroalkyl chains. Preincubation with aminobenzotriazole, but not pyrazole, inhibited the formation of metabolites from all FTOHs, suggesting that their oxidation was catalyzed by P450, not alcohol dehydrogenase.

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## 1. Introduction

The discovery that blood of the general human population contained organic fluorine compounds was first established in 1968 [1]. At that time, it was hypothesized that the unknown contaminants were similar in structure to perfluorooctanoic acid (PFOA) [2], however, it was not until 2001 that unambiguous identification and quantification of PFOA was reported in human blood by high pressure liquid chromatography tandem mass spectrometry (HPLC/MS/MS) [3]. Prompted by the detection of longer chained perfluoroalkyl carboxylates (PFCAs) in wildlife samples [4,5], Kuklenyik et al. [6] recently demonstrated that the blood of American adults is also contaminated with a homologous series of PFCAs ( $\text{CF}_3(\text{CF}_2)_y\text{COO}^-$ , where  $y = 7-10$ ), including perfluorononanoate (PFNA,  $y = 7$ ), perfluorodecanoate ( $y = 8$ ), perfluoroundecanoate ( $y = 9$ ), and perfluorododecanoate ( $y = 10$ ). Surprisingly, the exposure sources for all of these substances are not understood.

The US Environmental Protection Agency has appealed for data regarding the sources of PFOA due to the risk of adverse developmental effects in human offspring [7]. Chronic human exposure to PFCAs is also of concern given the non-genotoxic tumorigenicity of PFOA in rats [8] and the inhibitory effect of PFOA and perfluorodecanoate on gap-junction intercellular communication [9]. The toxicological information pertaining to PFCAs is limited largely to PFOA and perfluorodecanoate, however, PFNA and perfluoroundecanoate produce effects that are similar to those elicited by PFOA and perfluorodecanoate [10,11]. The half-life of PFOA in human blood is estimated to exceed 4 years [12], and although the pharmacokinetics of longer PFCAs has not been examined in humans, longer perfluoroalkyl chains equate to longer elimination half-lives in experimental animals [13–15]. It can be generalized that all PFCAs resist catabolism

and phase II conjugation, and are poorly excreted in humans.

To mitigate any future identifiable risks associated with PFCAs, it is necessary to understand their source(s) of exposure. Human PFCA exposure may result from two broad hypothetical scenarios: (i) direct exposure to PFCAs in commercial products, household dust, or ingestion of food and water containing PFCAs, or alternatively, (ii) via similar exposure routes to precursor molecule(s) that can be metabolized to PFCAs. The only documented direct use of long-chain PFCAs, other than PFOA, is as polymerization aids in fluoropolymer processing [16], but they are also fluoropolymer thermolysis products [17]. These sources may result in some human exposure to PFCAs but these are not examined here. Rather, based on the widespread detection of a series of fluorotelomer alcohols (FTOHs;  $\text{CF}_3(\text{CF}_2)_x\text{C}_2\text{H}_4\text{OH}$ ; where  $x = 3, 5, 7, 9$ ) in ambient air [18,19], we hypothesize that the later route of exposure is responsible, at least in part, for current human PFCA concentrations. For example, it is established that 8:2 FTOH (e.g. where  $x = 7$ ) is metabolized to PFOA in rats [20], however, it is unknown to what extent other PFCAs are also formed from FTOHs. It is also not known if reactive intermediates are formed, and hence if there are any additional adverse health consequences to be expected upon FTOH exposure.

FTOHs belong to a class of telomerized fluorochemicals, having an estimated global production of  $5 \times 10^6$  kg/year [21], that find use in a diverse range of commercial and industrial applications including paints, coatings, polymers, adhesives, waxes, polishes, electronics, and caulks [22]. Presumably as a result of their widespread use, 6:2, 8:2, and 10:2 FTOH (e.g.  $x = 5, 7,$  and  $9$ ) are now widespread in the North American atmosphere and human exposure can be expected. Although the magnitude of human exposure to FTOHs has not been assessed, their widespread distribution in ambient air warranted a comprehensive

examination of their metabolic fate. Herein, we report results from *in vivo* and *in vitro* metabolism studies of the metabolic products and pathways for a series of FTOHs. Results from tandem mass spectrometry experiments are reported here for an 8:2 FTOH exposed rat to validate the early metabolite identification work of Hagen et al. [20], but we focus on results from various isolated rat hepatocyte incubations dosed with 8:2 FTOH, or its synthesized intermediates, in an effort to elucidate the metabolic pathways leading to several novel and reactive metabolites. The respective metabolite profiles for 4:2, 6:2, 8:2, and 10:2 FTOH are also compared in isolated rat hepatocytes.

## 2. Experimental procedures

### 2.1. Chemicals

HPLC grade methanol and acetonitrile, ammonium acetate (>97%), 2,4-dinitrophenylhydrazine (97%; DNPH), PFOA (98%), PFNA (97%), pyrazole (98%) and aminobenzotriazole (98%) were purchased from Aldrich Chemical Co. Hydrochloric Acid was obtained from EM Science (Gibbstown, NJ, USA). 4:2 (97%), 6:2 (97%), 8:2 (97%), and 10:2 (97%) FTOH were purchased from Oakwood Products, Inc. (West Columbia, SC). The major impurity of 8:2 FTOH was the allylic alcohol ( $\text{CF}_3(\text{CF}_2)_6\text{CF}=\text{CHCH}_2\text{OH}$ ) [23], and all FTOHs were used without further purification. Other fluorochemical standards, including 8:2 fluorotelomer carboxylic acid (8:2 FTCA), 8:2  $\alpha,\beta$ -unsaturated fluorotelomer acid (8:2 FTUCA) and fluorotelomer aldehyde (8:2 FTAL) were synthesized in our laboratory according to methods described elsewhere [23], and their purities were all >95% [23]. The 8:2  $\alpha,\beta$ -unsaturated fluorotelomer aldehyde (8:2 FTUAL) was also synthesized in this study for spectral comparison to a metabolite but was not purified or used for pathway elucidation. A small portion of this impure 8:2 FTUAL material was added to a 10 mL saturated solution of  $\text{Na}_2\text{CO}_3$  containing 200 mg of GSH and left to react overnight. The dehydrofluorinated GS-8:2 FTUAL conjugate was confirmed by HPLC/MS/MS, at  $m/z$  728, as described in the results.

An authentic standard of the DNPH derivative of 8:2 FTAL was prepared by combining 1 mL of 0.018 M 8:2 FTAL in methanol with 0.9 mL of

0.018 M DNPH in hydrochloric acid, and allowing the reaction to proceed overnight at room temperature. The reaction product was extracted into diethyl ether and back-extracted with acidified water to remove unreacted DNPH. The ether phase was filtered through anhydrous  $\text{MgSO}_4$  and evaporated to dryness to yield a yellow powder. Direct MS infusion of this product in acetonitrile, in negative ion mode, revealed a pseudomolecular ion at  $m/z$  641 corresponding to the mass of the expected hydrazone derivative.

### 2.2. *In vivo* rat study

The purpose of this experiment was to validate the observations of Hagen et al. [20] before proceeding with more extensive hepatocyte experiments. In one experiment, male Sprague–Dawley rats were dosed with either 400 mg/kg 8:2 FTOH dissolved in corn oil ( $n=1$ ), or with corn oil only ( $n=1$ ) by intraperitoneal injection. The animals were anaesthetized at 6 h and samples of blood collected. The blood was centrifuged immediately and the plasma fraction collected and frozen until time of analysis. The choice(s) of dose, delivery mode, and sample collection times were chosen to reproduce the methods of Hagen et al. [20]. Samples of liver and kidney were also taken from both rats and frozen until analysis.

### 2.3. *In vitro* hepatocyte studies

Male Sprague–Dawley rats (275–300 g) were fed standard chow diet and water *ad libitum*. The animals were maintained two per cage in ventilated plastic cages over hardwood bedding in our central facilities (12 air exchanges per hour) at  $22 \pm 1^\circ\text{C}$ , 50–60% relative humidity and a 12-h light–12-h dark cycle. The animals were held under these conditions for 3–10 d prior to hepatocyte preparation.

Hepatocytes were isolated from rat liver perfused with collagenase as described previously by Mold us et al. [24]. Isolated hepatocytes were suspended in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM HEPES (10 mL,  $10^6$  hepatocytes  $\text{mL}^{-1}$ ) in continuously rotating round-bottomed 50 mL flasks and incubated under an atmosphere of 20%  $\text{O}_2$ , 75%  $\text{N}_2$ , and 5%  $\text{CO}_2$  in a water bath at  $37^\circ\text{C}$ . Hepatocytes were preincubated for 15 min prior to the addition of test chemicals or enzyme inhibitors. A preincubation period of

1 h followed the addition of enzyme inhibitors before test compounds were added to the suspensions.

To determine the metabolic products of FTOHs, 4:2, 6:2, 8:2, and 10:2 FTOH were added to isolated hepatocytes. To elucidate the pathways of metabolism we focused on 8:2 FTOH due to the wider availability of authentic standards (e.g. known and hypothesized metabolic intermediates) in our laboratory. Test substances used in pathway elucidation included 8:2 FTOH, 8:2 FTAL, 8:2 FTCA, 8:2 FTUCA, PFOA, and PFNA. The concentration of test substances used in hepatocyte suspensions ranged from 20 to 200  $\mu\text{M}$  in various experiments, and a control incubate was used on every day to monitor baseline cell toxicity and contamination with background perfluoroalkyl contaminants. No toxicity was observed at these concentrations for any chemical, as determined by Trypan blue exclusion, in the experimental time frame (2–4 h). Samples (1–2 mL) were collected by pipette and added to polypropylene tubes containing an equal volume of organic solvent, sodium carbonate (pH 10), or acid at various times throughout the course of these experiments. Samples were either processed immediately or frozen until analysis.

#### 2.4. Sample preparation

Acid metabolites were ion-pair extracted from blood, liver, kidney, or hepatocytes using tetrabutyl ammonium hydrogen sulfate as previously described [5]. Glucuronide and sulfate conjugates were detected by the same procedure, and also by 1:1 addition of methanol to an aliquot of the hepatocyte suspension, followed by centrifugation and filtration. GSH conjugates were examined for by extraction of the hepatocyte suspension with acetonitrile, and subsequent filtration before HPLC/MS/MS analysis. Aldehydes were derivatized to DNPH as described below. To avoid contamination of samples with perfluoroalkyl compounds, contact with polytetrafluoroethylene was eliminated at all stages of sample preparation [25]. Polypropylene vials were used for HPLC analysis that did not contain a polytetrafluoroethylene liner.

#### 2.5. HPLC/MS/MS identification of 8:2 FTOH and metabolites

All compounds were identified using reversed phase chromatography on a Genesis C8 column

(2.1 mm  $\times$  50 mm, Jones Chromatography, Lakewood, CO, USA) and mass spectral detection using a Micro LC (Micromass, Manchester, UK) triple quadrupole mass spectrometer equipped with an electrospray source operating in negative ion mode. Water, methanol, or acetonitrile solvents (10 mM ammonium acetate) were delivered at a total flow rate of 250  $\mu\text{L min}^{-1}$  by a Waters 600 controller using a linear gradient elution program as described previously [5]. Samples were injected (10–20  $\mu\text{L}$ ) using a Waters 717 Plus Autosampler (Waters, Milford A, USA). Data was acquired in full scan MS mode, or in MS/MS mode (daughter scan, parent scan, or multiple reaction monitoring). 8:2 FTOH was detected as the acetate adduct (i.e.  $[M + 59]^-$ ) in negative ion mode. Aldehydes were detected as the hydrazone derivative as described below.

#### 2.6. Aldehyde derivatization and identification

Aldehydes in hepatocyte samples were detected by HPLC/MS/MS as the respective hydrazone derivative following reaction with DNPH. A 2 mL sample of hepatocyte incubate was removed, mixed immediately with 2 mL of 0.018 M DNPH in hydrochloric acid, vortexed in a polypropylene tube and left to react for 12–24 h at room temperature before analysis. The solution was extracted with 10 mL of diethyl ether, blown to dryness under nitrogen, and taken up in 1 mL of acetonitrile. Reversed phase chromatography of the hydrazones was performed using an acetonitrile:water gradient elution program and with MS/MS detection by multiple reaction monitoring. Initial HPLC conditions were 40% acetonitrile: 60% water, and a linear gradient ramped to 100% acetonitrile over 7 min and was held for 5 min before reverting to initial conditions. Cone voltage was always 25 V and collision energy was always 20 eV.

### 3. Results

#### 3.1. Identification of acid metabolites

Mass spectrometric analysis of hepatocyte or rat tissue extracts confirmed the three acid metabolites previously reported by Hagen et al. for 8:2 FTOH [20]: PFOA, 8:2 FTCA, and 8:2 FTUCA (Fig. 1). Confirma-

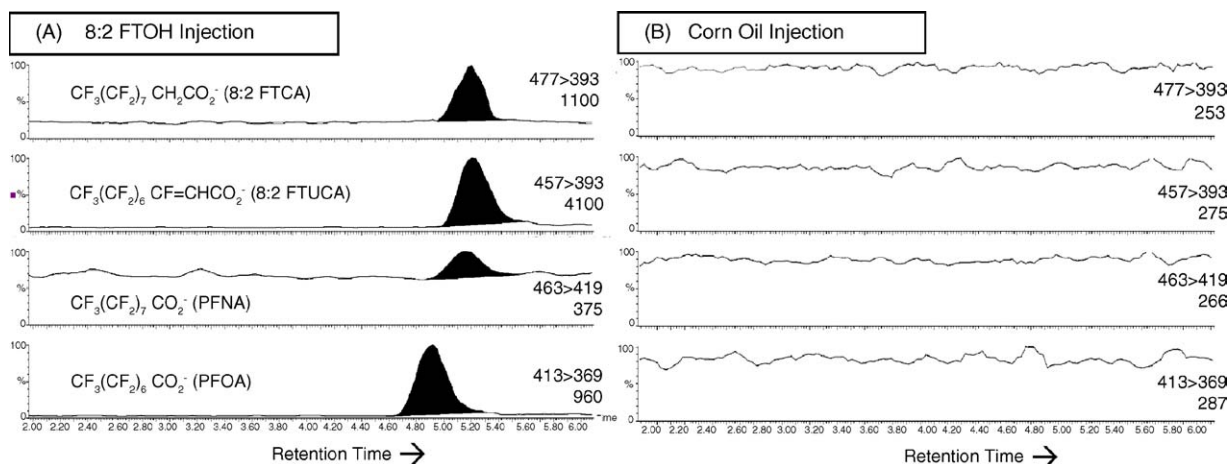


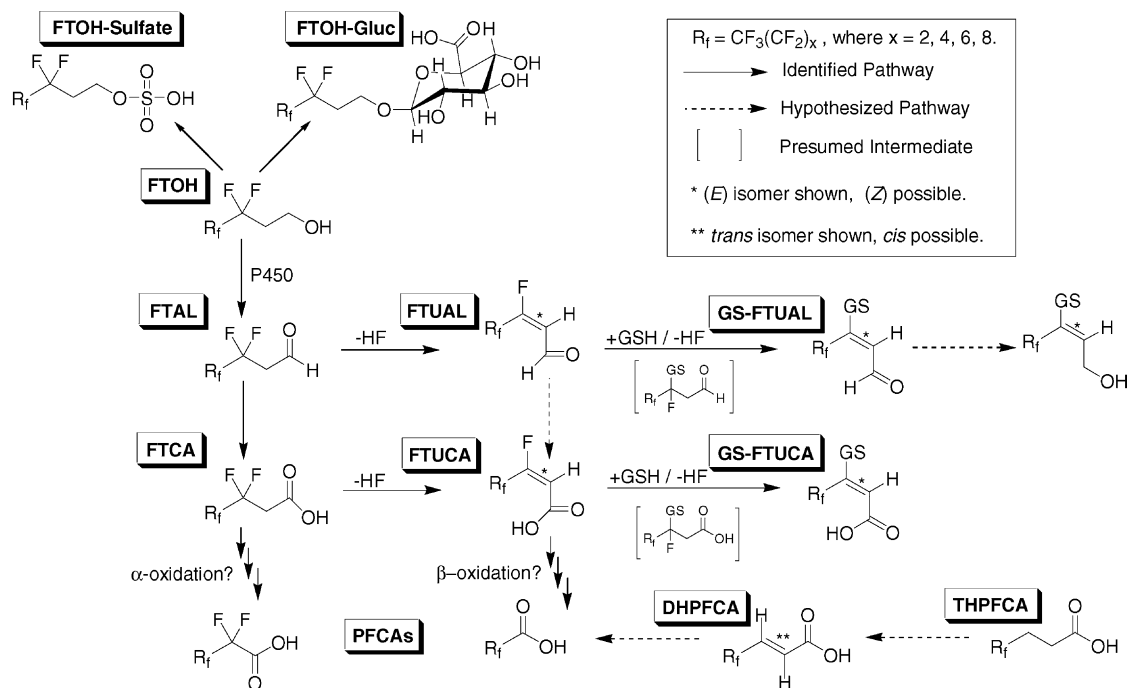
Fig. 1. HPLC/MS/MS multiple reaction monitoring chromatograms of some fluorinated acid metabolites identified in rat plasma for animals exposed to (A) 8:2 FTOH in corn oil or (B) corn oil only. The precursor  $\rightarrow$  product mass transition (e.g. 477 > 393), y-axis scale height, and chemical structures are shown on each chromatogram.

tion was based on retention time and product ion spectra that matched those of authentic standards. These acids were formed quickly from 8:2 FTOH and were confirmed in rat tissues (blood, liver, kidney) 6-h post dose, and also in isolated hepatocytes incubated for 1–3 h with 8:2 FTOH. A novel metabolite, PFNA, was also confirmed *in vivo* (Fig. 1) and *in vitro*, albeit the concentration was approximately 10-fold less than all aforementioned acids. Incubation of hepatocytes with 8:2 FTCA produced 8:2 FTUCA, PFOA, and PFNA, while incubations of hepatocytes with 8:2 FTUCA produced only PFOA (i.e. no PFNA). Incubates of 4:2, 6:2, or 10:2 FTOH resulted in the same metabolites at the same relative concentrations, differing only in perfluoroalkyl chain-length (Scheme 1).

A molar balance performed in triplicate indicated that the quantifiable acid products contributed only a minor amount to the total oxidation of 8:2 FTOH. After a 4 h incubation with 18  $\mu\text{M}$  8:2 FTOH, 78% of the parent material had been biotransformed. 8:2 FTCA (2.9%), 8:2 FTUCA (4.1%), PFOA (1.4%), and PFNA (<0.2%) combined to account for only 8.5% of the transformed fraction. A control experiment in dead cells indicated that 8:2 FTOH was stable and did not volatilize from the incubate during the molar balance experiment. In all FTOH incubates, lower concentrations of shorter FTCAs, FTUCAs, and a homologous series of shorter PFCAs were also observed, but these

did not contribute significantly to the molar balance and may have arisen from shorter FTOH impurities in each of the chemical standards. Hepatocytes incubated with 8:2 FTCA and 8:2 FTUCA showed that these substrates were metabolized much more slowly than 8:2 FTOH; less than 10% of the parent material was biotransformed in a 2 h period in both circumstances. Unlike experiments with the 8:2 FTAL and 8:2 FTOH, however, the molar balance of the transformed fractions exceeded 80% for 8:2 FTCA and 8:2 FTUCA based on quantifiable polyfluoroalkyl and perfluoroalkyl acids. Incubations performed with PFOA or PFNA showed that PFOA and PFNA concentrations were constant throughout the course of these experiments, thus providing no evidence of metabolism or reactivity for PFCAs.

Preincubation of hepatocytes with 100  $\mu\text{M}$  or 1 mM pyrazole (an alcohol dehydrogenase inhibitor) did not affect the quantity of acids measured in hepatocytes incubated with FTOHs. However, preincubation of hepatocytes with 1 mM aminobenzotriazole (non-specific P450 inhibitor [26]) led to a drastic decrease in metabolites for all FTOHs. For example, with 8:2 FTOH we observed a 9.1-fold reduction in FTCA, a 260-fold reduction in FTUCA, a 9.9-fold reduction in PFOA, and all other acids were not detectable. Neither pyrazole nor aminobenzotriazole affected cell mortality at the concentrations used.



Scheme 1. Generalized FTOH metabolic products and pathways in isolated rat hepatocytes. GSH conjugates, THPFCA, and DHPFCA were only examined in 8:2 FTOH incubates but are shown as generalized structures.

Two additional polyfluorinated acids were detected in isolated hepatocytes incubated with 8:2 FTOH (not examined *in vivo*, or with other FTOHs). While a lack of authentic standards prevented their absolute quantification and confirmation, their identities are discussed here and their concentrations are estimated to be no greater than for PFOA based on instrumental response. The first of these acids yielded a product spectrum identical to the spectrum reported in Wang et al. [27] (after correcting for the lack of a radio-labelled carbon) corresponding to 2*H*,2*H*,3*H*,3*H*-perfluorodecanoic acid (Fig. 2A); referred to hereafter as tetrahydroperfluoroalkyl carboxylate (THPFCA). The second polyfluorinated acid has never been reported previously and was assigned the formula  $C_{10}F_{15}H_2O_2^-$  based on the ion observed at  $m/z$  439. Given the neutral loss of 44 (i.e.  $CO_2$ ) and the strong product ion at  $m/z$  369 (i.e.  $[CF_3(CF_2)_6]^-$ ), the structure of this metabolite is presumed to be 2*H*,3*H*-dihydroperfluorodecenoic acid  $CF_3(CF_2)_6CH=CHCO_2^-$  (Fig. 2B); referred to hereafter as dihydroperfluoroalkyl carboxylate (DHPFCA).

It was not determined whether DHPFCA was a *cis* or *trans* isomer.

### 3.2. Identification, behaviour, and pathways of aldehydes

Aldehyde metabolites were identified in isolated rat hepatocytes incubated with each FTOH (e.g. 4:2, 6:2, 8:2, and 10:2 FTOH in individual experiments), but results are described in detail here for the model compound 8:2 FTOH. Because aldehydes do not produce strong signals under electrospray ionization and may be unstable in solution, the hepatocyte medium was sampled at four time intervals (30 min, 1 h, 2 h, 4 h) and immediately reacted with DNPH to form stable hydrazone derivatives. An authentic synthesized standard of the hydrazone derivative of 8:2 FTAL ( $m/z$  641) yielded a product ion at  $m/z$  163 (Fig. 3A), indicative of an aldehydic carbonyl [28]. A precursor scan experiment for  $m/z$  163 in extracts of hepatocytes incubated with 8:2 FTOH revealed two distinct chromatographic

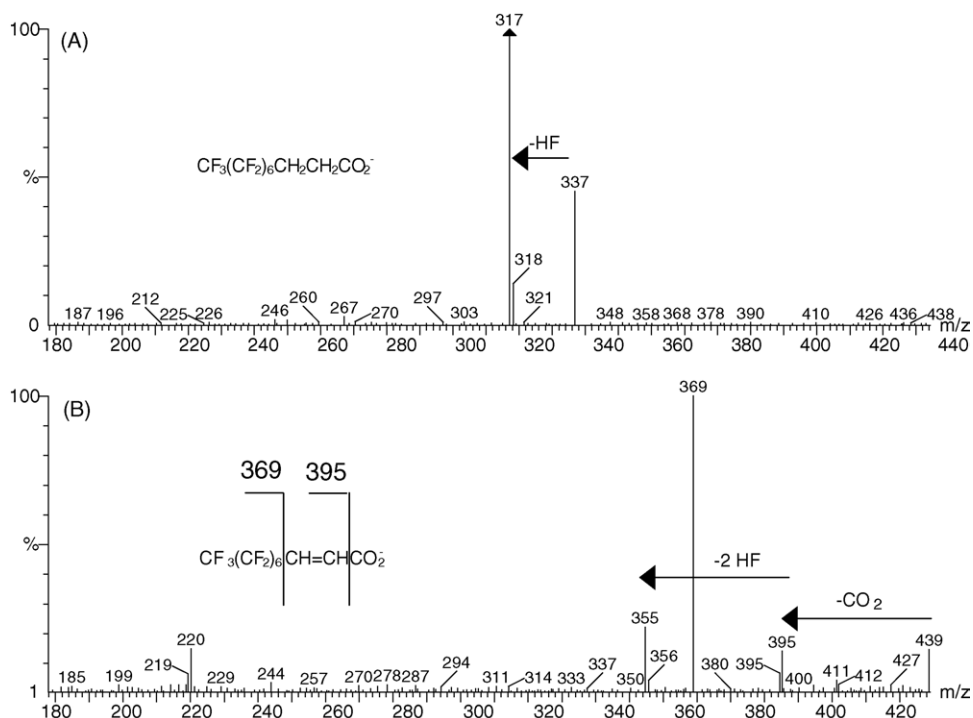


Fig. 2. Product ion spectra of (A)  $m/z$  441 and (B)  $m/z$  439, in rat hepatocytes incubated with 8:2 FTOH. These metabolites were assigned structures corresponding to polyfluorinated acids, specifically (A) tetrahydroperfluorodecanoic acid and (B) dihydroperfluorodecenoic acid. The same spectrum as in (A) was also published by Wang et al. [27] for the same metabolite containing a  $\text{C}^{14}$  label, and accurate mass measurement confirmed that it contained 4 hydrogen atoms, although the placement of these remains tentative.

peaks corresponding to precursor ions at  $m/z$  641 and 621. Neither peak was present in control hepatocytes derivatized with DNPH. The first of these peaks at  $m/z$  641 corresponded to the mass of the expected 8:2 FTAL derivative, and produced a product spectrum that matched the authentic standard product spectrum (i.e. compare Fig. 3A and B). The later eluting peak at  $m/z$  621 produced a product ion spectrum that was very similar to 8:2 FTAL, except that only one neutral loss of HF was evident for the deprotonated molecular ion (Fig. 3C). Although no authentic standard was available for comparison, the overall mass spectral evidence strongly suggested that this metabolite was the 8:2 fluorotelomer  $\alpha,\beta$ -unsaturated aldehyde (8:2 FTUAL).

To determine if 8:2 FTUAL was simply an artefact of the DNPH derivatization conditions, a control experiment was conducted by reacting DNPH directly with the authentic 8:2 FTAL standard. Based on relative instrument response, less than 1% FTUAL was

detected in the synthesized standard of 8:2 FTAL, indicating that the derivatization conditions could not explain the large amount of observed 8:2 FTUAL. Freshly isolated hepatocytes incubated directly with 8:2 FTAL yielded large amounts of 8:2 FTUAL in less than 30 min, indicating that 8:2 FTAL was its precursor. However, 8:2 FTUAL was also produced in significant quantities by incubating 8:2 FTAL with dead hepatocytes (100% mortality, determined by Trypan blue exclusion), or by incubation of 8:2 FTAL with sterile buffer or pure water at  $37^\circ\text{C}$ . In pure water, 8:2 FTAL diminished by 93% in 90 min, and by 99.9% in sterile buffer under the same conditions. Response of the 8:2 FTUAL increased proportionally up to 60 min, followed by a subsequent decrease at 90 min. These observations explain why when 8:2 FTOH or 8:2 FTAL were incubated with freshly isolated, or dead hepatocytes, neither the 8:2 FTAL or its unsaturate (8:2 FTUAL) could be detected after a 2 h period. A simi-

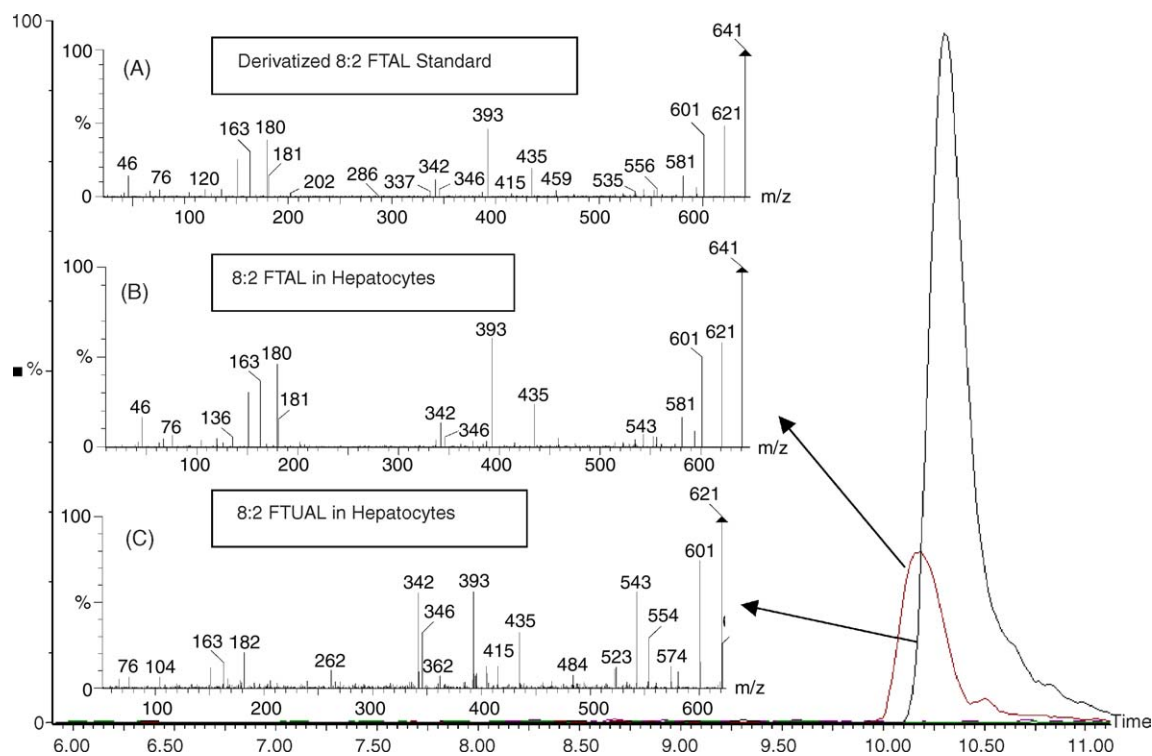


Fig. 3. Product ion HPLC/MS/MS chromatogram of aldehydes in an isolated hepatocyte extract derivatized with DNPH following a 1 h incubation with 8:2 FTOH, and also showing (A) a product ion scan of  $m/z$  641 obtained by direct infusion of an authentic hydrazone standard of 8:2 FTAL (i.e. derivatized to DNPH), (B) a product scan of the 641  $m/z$  peak, and (C) a product scan of the 621  $m/z$  peak. Spectrum B was identical to A, confirming the detection of 8:2 FTAL, while interpretation of spectrum C suggested the presence of the unsaturated aldehyde, 8:2 FTUAL.

lar experiment performed with the acid metabolite, 8:2 FTCA, showed no evidence for non-enzymatic dehydrofluorination over the time course of the experiment (3 h).

To help elucidate the overall FTOH metabolic pathway (Scheme 1), 8:2 FTAL (40  $\mu$ M) was incubated with hepatocytes for 2 h to determine its respective metabolites. No trace of 8:2 FTAL or 8:2 FTUAL was detectable after 2 h, but acid metabolites included small amounts of PFOA, PFNA, 8:2 FTCA, and 8:2 FTUCA. These were quantified but the molar balance of the acid products was low (<10%), suggesting that oxidation to carboxylic acids was not the primary fate for the aldehyde.

### 3.3. Identification of phase II conjugates

Targeted analysis of hepatocyte (in vitro) and rat liver (in vivo) sample extracts indicated the presence

of two previously unreported 8:2 FTOH metabolites, the corresponding *O*-glucuronide and *O*-sulfate. These species may play a role in excretion or enterohepatic recirculation of FTOHs in vivo. Identification was based on observation of the expected molecular ion in full scan MS, and diagnostic interpretation of their product ion spectra. For the 8:2 FTOH-glucuronide, the expected molecular ion appeared at  $m/z$  639 (i.e.  $[M - H]^-$ ) and yielded product ions corresponding to glucuronate ( $m/z$  193) and its dehydrate ( $m/z$  175) (Fig. 4A). For the sulfate, the expected pseudomolecular ion was detected at  $m/z$  543 and yielded an abundant product ion at  $m/z$  97, corresponding to sulfate (Fig. 4B). As with most polyfluorinated metabolites, a neutral loss of 20 was apparent in the product spectra of the glucuronide and sulfate at  $m/z$  619 and 523, respectively, corresponding to neutral loss of HF in both instances. The corresponding FTOH-sulfate for each FTOH was detected and confirmed by MS/MS in

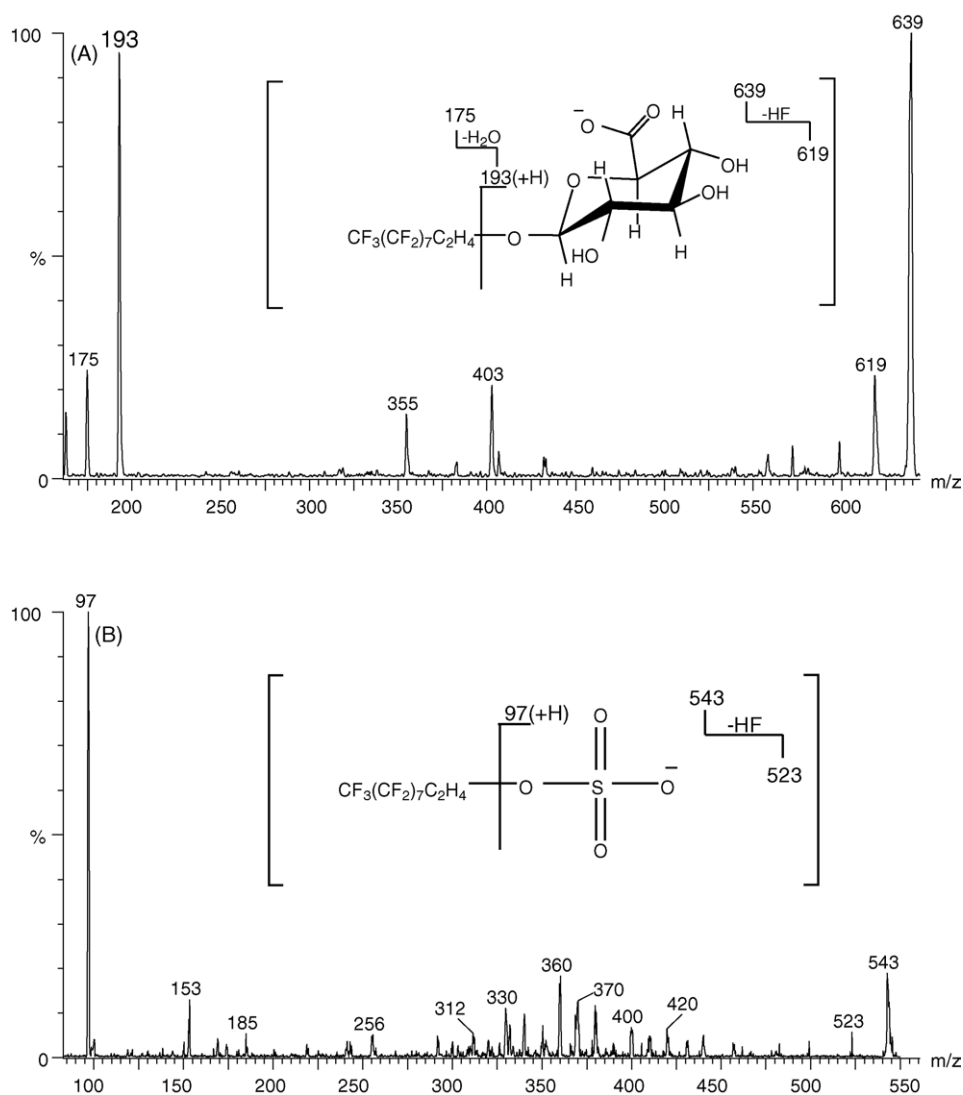


Fig. 4. Product spectra of metabolites detected at *m/z* 639 and 543 in hepatocytes incubated with 8:2 FTOH. Product ions were highly diagnostic at these were identified as the (A) *O*-glucuronide and (B) *O*-sulfate, respectively. Analogous spectra were also collected for the glucuronides and sulfates of 4:2, 6:2, and 10:2 FTOH (data not shown).

separate hepatocyte incubations, whereas the FTOH-glucuronide was only confirmed in 4:2, 6:2, and 8:2 FTOH incubations. Aminobenzotriazole preincubation caused a major increase in the FTOH-glucuronide and FTOH-sulfate response for all FTOH incubates, as determined by HPLC/MS/MS response. This result paralleled the significant decrease in acid oxidation products described earlier.

Three GSH conjugates were detected in isolated rat hepatocytes incubated with 8:2 FTOH (not investigated with other FTOHs). Their identification as GSH conjugates was based on precursor ion scans for *m/z* 306, 272, and 254, which are highly specific and diagnostic ions produced by dissociation of the GSH moiety. This approach identified *m/z* 728, 744, and 730 as GSH conjugates, and interpretation of their

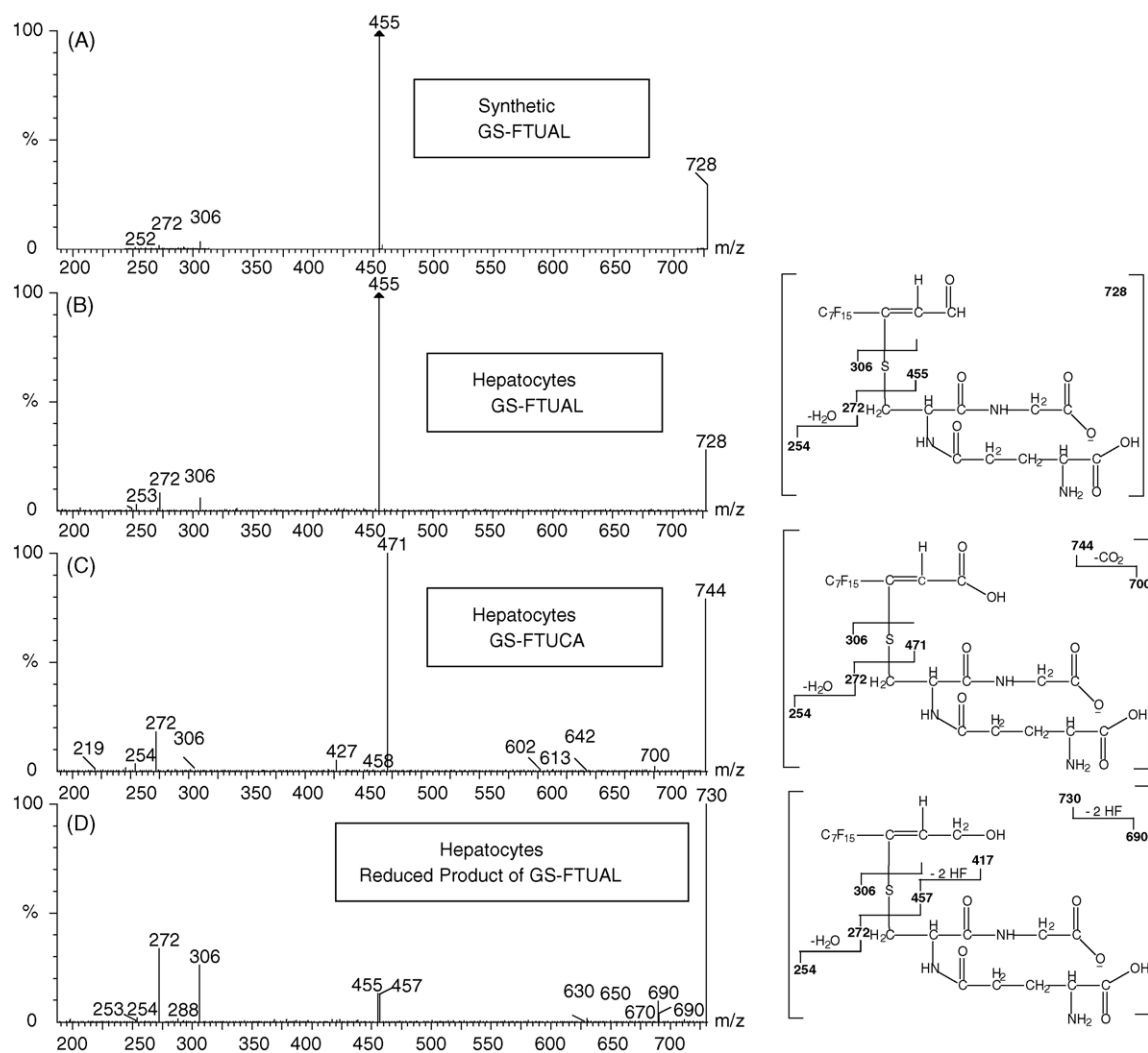


Fig. 5. Product ion spectra for (A) an authentic standard produced by reaction of 8:2 FTAL with GSH. Product ion spectra interpretation for three GSH conjugates observed in rat hepatocytes incubated with 8:2 FTOH and identified as (B) the dehydrofluorinated GSH conjugate of 8:2 FTAL (i.e. same as (A)), (C) the dehydrofluorinated GSH conjugate of 8:2 FTUAL, and (D) the alcohol product formed by reduction of (B).

product spectra suggested that  $m/z$  728 and 744 were the dehydrofluorinated 1,4 addition products of both unsaturated metabolites: GS-FTUAL and GS-FTUCA, respectively (Fig. 5B and C). A product having a  $m/z$  of 728 was synthesized by reacting an authentic (yet impure) standard of 8:2 FTUAL with GSH in sodium bicarbonate buffer, and its product spectrum matched the spectrum for the metabolite detected in hepatocytes at  $m/z$  728 (i.e. compare Fig. 5A and B). GS-FTUCA was also observed in an incubation

of 8:2 FTCA, whereas GS-FTUAL was not observed (Scheme 1). There was no spectral evidence for any related terminal-carbon GSH addition products.

Based on product spectrum interpretation (Fig. 5D), the third GSH conjugate ( $m/z$  730) was determined to be the reduced product of GS-8:2 FTUAL. In incubations of 8:2 FTOH containing 1 mM aminobenzotriazole the instrumental response of this alcohol was reduced 26-fold, relative to uninhibited cells, indicating that it was not a direct conjugation product formed between

GSH and the allylic alcohol impurity in the 8:2 FTOH test substance. Furthermore it could not be detected in incubations of 8:2 FTCA, indicating that it must have been produced through metabolism of the aldehyde, 8:2 FTAL. This partially accounts for the low molar balance of quantifiable (e.g. acid) products observed in 8:2 FTAL incubates.

## 4. Discussion

### 4.1. Acid metabolites and unaccounted molar balance

The *in vivo* detection of PFOA, 8:2 FTCA, and 8:2 FTUCA validates, with mass spectral evidence, the early 8:2 FTOH metabolite identification work of Hagen et al. performed by gas chromatography of methyl ester derivatives and using a helium microwave plasma detector [20]. Some fraction of the mass balance attributed to FTUCA in this study may result from metabolism of the allylic alcohol impurity (maximum 3%, based on purity), but certainly FTUCA is also a metabolite of 8:2 FTOH, as evidenced by its detection as a major metabolite in incubations with the intermediate species, FTCA. Hagen et al. specifically looked for the production of PFNA from 8:2 FTOH but concluded that none was detectable in rat plasma. The detection of PFNA herein (*in vivo* and *in vitro*) does not contradict this earlier finding, rather, this discrepancy is presumably due to the lower detection limits of our analytical method. Although no detection limits were reported by Hagen et al., our method allows detection on the order of 1 pg injected for PFNA [29]. DHPFCA and THPFCA were also not reported by Hagen et al, however, one of these could explain the unknown “peak z” in their chromatograms published in 1981 [20].

Although significant, the low molar balance for the quantifiable acid metabolites, including PFOA, indicates that they are not the major metabolic fate of FTOHs in rat hepatocytes. The unaccounted molar balance is at least partially explained by the five novel conjugates (three GSH, one glucuronide, and one sulfate), and to a lesser extent by DHPFCA and THPFCA. Although we synthesized two polyfluorinated acids to arrive at the partial mass-balance presented here, we were unable to quantify these additional unexpected

metabolites due to a general lack of commercially available chemical standards and/or appropriate synthetic starting material, including pure 8:2 FTUAL (a small amount of impure 8:2 FTUAL was generated and the corresponding GSH synthesized for spectral matching in Fig. 5A, but this could not be purified to allow quantification). The quantifiable molar balance in hepatocyte incubations of polyfluorinated acids (8:2 FTCA and 8:2 FTUCA) are reasonable (>80%), whereas the quantifiable molar balance in incubations of either 8:2 FTOH or 8:2 FTAL are poor (i.e. less than 10% in both circumstances). While it is possible that these five novel conjugates may explain the unaccounted molar balance, as we argue later on, future studies should also consider non-specific reactions of the electrophilic metabolites (particularly 8:2 FTUAL) with endogenous biological macromolecules or other common cellular nucleophiles.

### 4.2. Possible roles of $\alpha$ - and $\beta$ -oxidation

Although the amount of PFNA observed was negligible relative to PFOA, its presence suggests that  $\alpha$ -oxidation may be occurring to some extent in the metabolic pathway of 8:2 FTOH. Such a pathway would presumably require  $\alpha$ -hydroxylation of 8:2 FTCA and subsequent oxidation yielding a perfluorinated alkenal, and eventually PFNA. None of these intermediates were detected in this study, however, neither were they targeted. PFNA was also observed in incubations with 8:2 FTCA, but not with 8:2 FTUCA, suggesting that  $\alpha$ -hydroxylation may be diminished by dehydrofluorination.

PFOA, 8:2 FTCA, and 8:2 FTUCA were first identified as biotransformation products of 8:2 FTOH in rats [20], and later in mixed microbial cultures [23] and activated sewage sludge [27]. Whereas Hagen et al. [20] and Dinglasan et al. [23] both suggested that  $\beta$ -oxidation was the probable mechanism for PFOA production, Wang et al. [27] argued that  $\beta$ -oxidation could not proceed through any known FTOH metabolite because each has too few hydrogen atoms to reduce the necessary number of NAD or FAD molecules. For example, 8:2 FTCA contains no hydrogen atoms at the  $\beta$ -position, and 8:2 FTUCA contains only one hydrogen atom at the  $\alpha$ -position. However, whether or not the reaction is catalyzed by a  $\beta$ -oxidation enzyme, we have shown here that 8:2 FTCA dehydrofluori-

nates in hepatocytes to yield 8:2 FTUCA by some mechanism that is analogous to the first step of the  $\beta$ -oxidation cycle involving a  $\Delta^2$  desaturation of fatty acyl-CoA by Acyl-CoA dehydrogenase. We are not aware of any previous studies that have examined the influence of  $\beta$ -carbon fluorination on the  $\beta$ -oxidation cycle, however, some important information may be drawn from studies performed with fluorinated dicarboxylate analogues of the citric acid cycle. For example, dehydrofluorination was observed for 2,2-difluorosuccinate (i.e.  $\text{CO}_2^- \text{CF}_2 \text{CH}_2 \text{CO}_2^-$ ), which in the presence of sub-mitochondrial particles yielded monofluorofumarate (i.e.  $\text{CO}_2^- \text{CF}=\text{CHCO}_2^-$ ) [30]. Therefore, some mechanism exists in mitochondria for the oxidation of polyfluorinated acids that does not necessarily require simultaneous reduction of FAD.  $\beta$ -Carbon hydration (e.g. by the  $\beta$ -oxidation cycle enzyme enoyl-CoA hydratase) is NAD-independent, and may proceed for 8:2FTUCA, presumably yielding a  $\beta$ -hydroxyfluoro intermediate analogous to the hydroxylation of monofluorofumarate to yield 2-fluoromalate (i.e.  $\text{CO}_2^- \text{CF}(\text{OH})\text{CH}_2\text{CO}_2^-$ ) by the citric acid cycle enzyme fumarate hydratase [30]. In the case of 2-fluoromalate, this was unstable and dehydrofluorinated non-enzymatically to yield oxaloacetate (i.e. also NAD-independent) [30]. Therefore, precedent exists for a feasible mechanism for a hydrated 8:2 FTUCA molecule to yield a  $\beta$ -ketoacyl substrate, which would then presumably yield acetyl CoA and PFOA-S-CoA via the enzyme thiolase. Although it is technically correct that 8:2 FTCA and 8:2 FTUCA cannot produce PFOA by  $\beta$ -oxidation per se, as this would require the reduction of FAD and NAD, it cannot be ruled out that they may be processed through analogous intermediates, and perhaps catalyzed by certain enzymes of the  $\beta$ -oxidation cycle in mitochondria.

As an alternative mechanism for PFOA production, Wang suggested that  $\beta$ -oxidation is more likely to occur through the novel metabolite, THPFCA (i.e. 2*H*,2*H*,3*H*,3*H*-perfluorodecanoic acid) [27]. While indeed this metabolite, as shown in Scheme 1, is expected to yield PFOA in one round of  $\beta$ -oxidation, the pathway for its production remains unknown and the hydrogen atoms are not necessarily on carbons 2 and 3, thus this suggestion remains tentative. However, this theory is supported by the identification of DHPFCA in this study (i.e. 2*H*,3*H*-perfluorodecanoic

acid), which is the expected intermediate in the first step of the  $\beta$ -oxidation cycle for THPFCA (i.e.  $\Delta^2$  desaturation mediated by Acyl CoA dehydrogenase). Further research should examine the pathways leading to THPFCA and DHPFCA.

#### 4.3. Aldehyde detection and reactivity

This is the first study to detect an unsaturated aldehyde metabolite in any FTOH biotransformation study. Dinglasan et al. previously provided evidence for trace amounts of 8:2 FTAL in a biodegradation experiment using GC/MS [23], but an unsaturated aldehyde was not observed. We hypothesized that 8:2 FTAL was a necessary intermediate in any hepatic pathway leading from the alcohol to the acid, and also that it may dehydrofluorinate by some mechanism analogous to dehydrofluorination of the carboxylic acid, 8:2 FTCA. Thus we purposely targeted both aldehydes herein assuming that they may be transient in solution and not suitable to direct analysis by LC/MS. Our experimental observations suggested that 8:2 FTAL was unstable in water, and dehydrofluorinated ( $\gg 90\%$  in 90 min) at a physiological temperature and pH to yield 8:2 FTUAL. 8:2 FTUAL itself was also transient, however, its fate is unknown and volatilization cannot be ruled out. Therefore, trapping these aldehydes as stable hydrazones was essential for their detection and analysis, and also partially explains why previous studies did not identify both aldehydes detected here. Identification of the non-enzymatic dehydrofluorination pathway, yielding 8:2 FTUAL from 8:2 FTAL, is important because it also provides an alternative pathway leading to the  $\alpha,\beta$ -unsaturated acid metabolite 8:2 FTUCA (Scheme 1). Dehydrofluorination was a common degradation pathway for many of the polyfluoroalkyl intermediates observed in this study, and was also a consistent and useful marker in tandem mass spectrometry used here for identification of polyfluorinated metabolites via neutral losses of 20 (i.e. HF) or 40 (i.e. 2 HF) mass units (Figs. 2–5).

The two  $\alpha,\beta$ -unsaturated metabolites are electrophilic substances based on the observation of their dehydrofluorinated GSH conjugates: GS-8:2 FTUAL and GS-8:2 FTUCA. Observation of GS-8:2 FTUCA in an incubation of 8:2 FTCA demonstrated that GSH reacted directly with the unsaturated acid, and that this was not an oxidation product of GS-8:2 FTUAL. Two

resonance structures can be drawn for each unsaturated metabolite, whereby the electrophilic centre can be situated on either the  $\beta$ - or carbonyl-carbon. We did not observe a GSH conjugate corresponding to reaction at the carbonyl-carbon, as sometimes occurs when strongly electron withdrawing groups are adjacent to the  $\beta$ -carbon such as two trifluoromethyl groups [31]. For addition of GSH, the overall effect of the  $\beta$ -carbon fluorine and adjacent  $C_7F_{15}$  moiety seems to be a strong potentiation of the  $\beta$ -carbon centred electrophile, and thus 1,4 addition. Conjugation of these electrophilic species to GSH probably aids their biliary excretion *in vivo*.

The identification of these GSH conjugates was complicated because of the unexpected dehydrofluorination of the 1,4 addition product (Scheme 1). Non-dehydrofluorinated conjugates were not detected in hepatocytes, nor in the synthesized material, suggesting that the 1,4 addition product is unstable. This may be of important toxicological consequence, because the resulting dehydrofluorinated GSH conjugates are themselves  $\alpha,\beta$ -unsaturated aldehydes that could, theoretically, react with a second nucleophile.

The fate of 8:2 FTUAL in isolated rat hepatocytes deserves further attention but can be compared to the state of knowledge regarding another electrophilic  $\alpha,\beta$ -unsaturated metabolite of similar size, 4-hydroxynonenal (HNE). The primary identified fate for HNE includes GSH conjugation and oxidation to 4-hydroxynonenic acid (HNA), while a minor fate involves reduction to the 1,4-diol [32,33]. For 8:2 FTUAL, we did not have a pure standard with which to perform fate experiments, yet its GSH conjugate was observed and the unsaturated acid oxidation product (i.e. FTUCA) was a prominent metabolite formed in 8:2 FTOH or 8:2 FTAL incubations. The reductive pathway for 8:2 FTUAL was not targeted in this study, but in any case the reduced product would likely be oxidized back to 8:2 FTUAL in an equilibrium process as suggested for HNE and its 1,4-diol [32]. Scheme 1 includes a hypothesized pathway leading from 8:2 FTUAL to 8:2 FTUCA, based on the analogous fate of HNE, yet this pathway remains to be tested.

A hepatocellular pathway suggested here for 8:2 FTUAL, which has not been observed for HNE in hepatocytes, involves reduction of GS-8:2 FTUAL to the corresponding GS-alcohol observed in 8:2 FTOH incu-

bations (Scheme 1 and Fig. 5D). This pathway is a major fate for GS-HNE in erythrocytes and intact heart, catalyzed by aldose reductase to form glutathionyl 1,4-dihydroxynonene [34,35], but we are unaware of any evidence for this pathway in hepatocytes to date. The reductive pathway observed here for GS-8:2 FTUAL may serve as a protective measure by preventing dissociation of the GSH conjugate to free aldehydes, and the catalytic role of aldehyde reductase should be examined.

There is also no evidence in the literature for reaction of GSH with HNA, the unsaturated acid metabolite of HNE, in any cell type. Surprisingly, mercapturate derivatives of GS-HNA were detected in urine of rats exposed to HNE about 10 years ago [36], yet we are not aware that its formation has been investigated in any cell type. Here, we have provided clear evidence for this conjugative pathway in rat hepatocytes, by direct incubation of 8:2 FTUCA and subsequent detection of its GSH conjugate. Therefore, oxidation of 8:2 FTUAL, or dehydrofluorination of 8:2 FTCA yielding 8:2 FTUCA, cannot necessarily be considered protective pathways and may lead to toxicity.

Given their reactivity with GSH, it is not unreasonable to hypothesize that 8:2 FTUAL and 8:2 FTUCA react with other cellular nucleophiles not identified here, such as cysteine, lysine, histidine, and nucleic acids. The low molar balance calculations reported here and in an FTOH biodegradation study with mixed microbes [23] may be partially explained by reaction with GSH and non-specific reactivity with organic matter. Such non-specific reactivity was hypothesized as an explanation for the low molar balance in *in vitro* hepatocyte studies with HNE [32], however, two years later it was determined that HNE reaction with proteins could only account for 3% of the molar balance in HNE incubations [33]. The non-specific reactivity of the unsaturated metabolites identified here should be examined because even a small amount of reactivity with proteins may have adverse toxicological consequences, and furthermore because the  $-CF_2CF=CH-$  moiety may result in a more electrophilic species compared to HNE, as evidenced by the unexpected reaction of FTUCA with GSH. Potential toxicological implications of FTUAL binding, based on knowledge accumulated for other direct acting alkylating agents, include the impairment of enzymes and genotoxicity [37].

#### 4.4. Enzymes involved in FTOH oxidation

Kaminsky et al. [38] demonstrated that trifluoroethanol metabolism was controlled by P450 2E1, and thus it may seem logical that FTOHs, also fluorinated alcohols, are metabolized by the same enzyme. However, trifluoroethanol is comparably small and also a strong inhibitor of alcohol dehydrogenase due to its strong acidity [39]. Therefore, because FTOHs are presumably much weaker acids (although no  $pK_a$  measurements have been made) we hypothesized that alcohol dehydrogenase may play a role in their metabolism. However the two experimental results reported here suggest that alcohol dehydrogenase plays an insignificant role, and that P450 catalyzes the initial oxidation to FTAL. Alcohol dehydrogenase may be incapable of metabolizing FTOHs, possibly because of the long hydrophobic polyfluoroalkyl chain.

#### 4.5. PFCAs and toxicological implications of human FTOH exposure

This work has demonstrated that FTOHs can be metabolized to PFCAs of various chain-lengths, depending on the starting chain-length, and thus exposure to FTOHs is a feasible explanation for the occurrence of long-chain PFCAs in human blood (e.g. 8:2 FTOH, 10:2 FTOH, and possibly 12:2 FTOH). However, the magnitude of human exposure to FTOHs is unknown and only future air monitoring efforts will determine if FTOH exposure can account for the low ng/mL PFCA concentrations in human blood [6]. For example, FTOHs are detectable in the outdoor atmosphere, but indoor air concentrations are currently unknown. Because the telomerization process, used to manufacture FTOHs, leads to a homologous series of even-carbon numbered chain-lengths [22], exposure to 6:2, 8:2, 10:2 FTOH, and possibly longer FTOHs presumably occurs simultaneously. Using outdoor FTOH concentrations ( $\sim 10\text{--}100\text{ pg/m}^3$  for the total of 6:2, 8:2, and 10:2 FTOH) [18,19], and average male and female rates of inhalation ( $\sim 15\text{ L/min}$  assuming 66% light activity and 33% resting daily) [40], a crude calculation suggests that the lower limit for daily human exposure is between 0.2 and 2 ng FTOH/day. This is assumed to be a lower limit because indoor air concentrations are probably higher than outdoors, and humans spend 90% of their time indoors. For example,

perfluorooctanesulfonamides, a related class of perfluorochemicals used in similar applications as FTOHs, are present in indoor air at concentrations that exceed outdoor air by 100-fold [41]. A qualitative measure of human exposure to FTOHs could also be determined by monitoring FTOH-glucuronides and FTOH-sulfates in human urine, or by measurement of oxidation intermediates in human blood or liver samples. It should be noted that another possible route of exposure to PFCAs, or FTCAs, is through their atmospheric deposition resulting from FTOH oxidation in the troposphere [42], and/or indirectly through drinking water or food.

Given the electrophilic metabolites identified in this study, further metabolic and toxicological investigations are warranted for FTOHs. There is currently little toxicological data available for FTOHs, but preliminary data on the toxicology of 8:2 FTOH indicated low acute toxicity from a single dose (e.g.  $LD_{50} > 2000\text{ mg/kg}$ ), negative results in an *in vivo* rat micronucleus assay, and no toxicity or increase in revertants in *Salmonella/Escherichia coli* reverse mutation assays [21].

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