COMPARATIVE METABOLISM AND DISTRIBUTION OF 8-2 FLUOROTELOMER ALCOHOL (FTOH) IN MALE, FEMALE AND PREGNANT MICE

by

WILLIAM MATTHEW HENDERSON

(Under the Direction of Mary Alice Smith)

ABSTRACT

Perfluorinated chemicals (PFCs) are anthropogenic and currently, there are no known natural sources of long-chain perfluorinated carboxylic acids (PFCAs) such as perfluorooctanoic acid (PFOA) or perfluorononanoic acid (PFNA) in the environment. The most plausible explanation for global dissemination is that stable acids like PFOA and PFNA are formed as metabolism and/or degradation products of several structurally-related homologues in the atmosphere, specifically 8-2 fluorotelomer alcohol (8-2 FTOH). An appropriate animal model is needed to fully elucidate the mechanisms of 8-2 FTOH metabolism and distribution in mammals. The objectives of this study were to 1) develop a method to simultaneously quantify known volatile and non-volatile metabolites of 8-2 FTOH, 2) determine the metabolism and distribution of 8-2 FTOH in adult male, female and pregnant CD-1 mice, and 3) determine the developmental effects to fetal and neonatal mice following *in utero* and post-natal exposure to 8-2 FTOH. Initially, a GC/MS method was developed and tested based on selected ion monitoring of

perfluorinated alkyl parent chain fragment ions (131, 169, 231 and 331 *m/z*). The method instrument detection limits are between 7.1 and 24.5 ng/mL extract, and the method quantification limits are below 50 ng/mL or ng/g in all matrices. In order to determine the pharmacokinetics of 8-2 FTOH metabolism, CD-1 mice received a gavage dose (10-30 mg 8-2 FTOH/kg BW) in a propylene glycol/water (1:1) vehicle on day 0 and timed-pregnant mice were dosed (30 mg 8-2 FTOH/kg BW) on gestational day (GD) 8. Animals were serially sacrificed and tissues excised for analysis via GC/MS. 8-2 FTOH was rapidly metabolized and no longer present by 48 hrs post-treatment (PT) and the estimated half-life ($t_{1/2}$) of 8-2 FTOH in serum of male and female mice was 6.2 and 5.8 hrs, respectively. In male mice, maximal PFOA concentrations in serum (992±38 ng/mL) and liver (902±27 ng/g) were at 12 hrs PT and decreased temporally for up to 120 hrs. The maximum concentrations in serum and liver of female (768±25 ng/mL and 699±38 ng/g) and pregnant mice (789±41 ng/mL and 673±23 ng/g) also occurred by 24 hrs PT. Using least-squares regression, the $t_{1/2}$ in male mice was 15.1 d and was comparable to female mice (14.9 d). However, during gestation (GD 8-18), the calculated $t_{1/2}$ was 31.3 d, but values after parturition were similar to non-pregnant females (12.9 d). PFOA appears in the developing fetus as early as 24 hrs PT and exposure of neonates to metabolites of 8-2 FTOH can occur both pre- and post-natally following a single oral maternal dose on GD8. Estimated PFOA $t_{1/2}$ in liver was 8.2, 9.2 and 28.4 d for male, female and pregnant mice, respectively. In conclusion, 8-2 FTOH is rapidly metabolized to PFOA in adult male, female and pregnant mice. Sex-mediated elimination is absent, but pregnant mice retain PFOA approximately 2x longer in serum than non-pregnant females suggesting that physiologic changes during pregnancy increase PFOA retention. Furthermore, maternal-fetal transfer of the

metabolites increases temporally throughout gestation suggesting that PFOA and PFNA accumulate in the fetuses and post-partum, neonates can likely be exposed via lactation.

INDEX WORDS: metabolism, distribution, perfluorinated compounds, perfluorooctanoic acid, PFOA, perfluorononanoic acid, PFNA, fluorotelomer alcohols, 8-2 FTOH, developmental toxicity, *in utero* exposure, CD-1 mouse, fetus, neonates

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DEDICATION

I would like to dedicate this and all my efforts to my family because without them, I would not be where I am today. Most importantly, this work is dedicated to my grandfather, W. Bennett (Benny) Malcom for showing me the man I strive to be. Papa, the journey to this end is just the beginning.

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CHAPTER 1

INTRODUCTION

The environmental and mammalian bioavailability of PFCs, such as perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS), is of concern because of their growing presence and persistence in global matrices (Giesy et al. 2001; Martin et al. 2004). These compounds have been shown to be distributed worldwide (Giesy and Kannan 2001), bioaccumulated (Tomy et al. 2004), persistent (Evans et al. 2005) and potentially toxic (Butenhoff et al. 2004; Kennedy et al. 2004; Lau et al. 2004). Historically, these chemicals were used in a wide variety of industrial and consumer products including inert insecticide formulations, industrial surfactants, and processes detailing carpet and apparel coatings (Dinglasan-Panlilio and Mabury 2006; Lehmler 2005; Prevedouros et al. 2006).

It has been hypothesized that many of these fluorinated compounds, namely the fluorotelomer alcohols (FTOHs), degrade or metabolize to the stable metabolites, PFOA and perfluorononanoic acid (PFNA) (Dinglasan et al. 2004; Martin et al. 2005; Wallington et al. 2006; Wang et al. 2005). Following release, 8-2 FTOH and structurally analogous compounds are likely transported via atmospheric processes and subsequently transformed into PFOA via both metabolism and biodegradation processes (Fasano et al. 2006; Kudo et al. 2005; Martin et al. 2005). FTOHs are used as precursor compounds in the production of fluorinated polymers and have been detected in the North American troposphere from 17-135 pg/m^3 (Stock et al. 2004). These fluorochemical precursors, with varying physiochemical properties, allow the

migration of PFOA and PFNA in the environment following subsequent environmental degradation or mammalian metabolism of 8-2 FTOH.

Human exposure to PFOA and PFNA has been demonstrated (Calafat et al. 2005; Calafat et al. 2006; Olsen et al. 2003a; Olsen et al. 2003b) and the United States Environmental Protection Agency (USEPA) has identified the probable human health concerns from exposure to these metabolites (USEPA, 2001); however, there is still considerable uncertainty surrounding the potential risks. In rodent and non-human primate models, PFCs have been shown to cause peroxisome proliferation. PFCs also affect the normal fatty acid metabolism occurring in the liver as well as microsomal and mitochondrial fatty-acid β-oxidation pathways (Kennedy et al. 2004; Kudo et al. 2000; Kudo et al. 2005). Other related toxic effects include the accumulation of triglycerides in the liver, hypertrophy and hepatomegaly, reduction in mitochondrial oxidative phosphorylation, and reduction of thyroid hormone availability (Adinehzadeh and Reo 1998; Gilliland and Mandel 1996; Goecke-Flora and Reo 1996; Kudo et al. 1999).

More importantly, numerous PFCs are developmentally toxic resulting in embryonic and post-natal death and growth retardation in rodents (Butenhoff et al. 2004; Lau et al. 2004; Thibodeaux et al. 2003). In the mouse, PFOA exposure during pregnancy produced dose-dependent effects on the number of resorptions, maternal and fetal weight gain, post-natal survival and growth deficits (Lau et al. 2006). Early gestational exposure has been associated with growth reduction, low body weight, and poor post-natal survival of pups (Lau et al., 2006).

Due to the increasing use of perfluorinated compounds in both industrial and consumer applications and the potential for adverse health effects, there is an increased need to investigate the human-health risks associated with exposure and to elucidate the possible exposure scenarios. To answer this, there were three main goals of this research 1) to develop a method

to simultaneously quantify known volatile and non-volatile metabolites, 2) to determine the metabolism and distribution of 8-2 FTOH in male, female and pregnant CD-1 mice and determine if it is a likely precursor compound for PFOA, and 3) determine the developmental effects of 8-2 FTOH following *in utero* and post-natal exposure. Because simultaneous quantification is needed for volatile and non-volatile perfluorinated chemicals (PFCs) in complex matrices, and to aid in answering goals 2 and 3, a GC/MS method was developed and tested based on selected ion monitoring of perfluorinated alkyl parent chain fragment ions.

Purpose of Study

The purpose of this study was to investigate the mammalian metabolism of the fluorochemical precursor, 8-2 FTOH in male, female and pregnant CD-1 mice elucidating the pathways associated with 8-2 FTOH metabolism and quantifying the subsequent distribution of the terminal metabolites, PFOA and PFNA. Furthermore, developmental toxicity studies were conducted to examine the effects of 8-2 FTOH and its subsequent metabolism to PFOA on both the developing fetus and neonate. To conduct these studies, a method was needed to allow for the concurrent analysis of volatile and non-volatile PFCs associated with 8-2 FTOH metabolism. The metabolic parameters of 8-2 FTOH have been sparsely studied in rats; however, limited information is available on metabolism of the 8-2 FTOH in an appropriate animal model. The results of this research contributes to the overall goal to develop a model system, including but not limited to, the entire chain of 8-2 FTOH metabolism in mammalian systems, that can be used to formulate structure activity relationships and the initiation of a PBPK model for 8-2 FTOH, PFOA and related perfluorinated compounds. This research will ultimately aid in developing a model to determine the continuum of environmental exposure to precursor compounds (8-2

FTOH) and whether subsequent mammalian metabolism of precursor compounds accounts for the concentration of PFOA and PFNA detected globally.

Scope of Dissertation

This dissertation includes a literature review of the state of the science of perfluorinated chemicals, namely 8-2 FTOH and PFOA. Due to the environmental and toxicological relevance of PFOA, it has been the focus of current literature and limited information is available on the toxicology and metabolism of 8-2 FTOH. Included in the literature review is a summary of the current knowledge detailing the environmental relevance of PFCs, a review of their unique physical and chemical properties that must be considered when developing analytical methodologies, environmental degradation and mammalian metabolism processes, and the toxicity of 8-2 FTOH and PFOA.

Following the literature review, chapter 3 describes a method for the simultaneous detection and quantification of 8-2 FTOH and its metabolites. This chapter is in press in Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences (Henderson et al., 2006). The following two chapters discuss the background, methods, and results of the metabolism and distribution experiments in male, female and pregnant mice. Chapter 4 describes results in the male CD-1 mouse and will be submitted to Toxicological Sciences. Chapter 5 has been accepted for publication in Toxicological Sciences and details the distribution of PFOA and PFNA in fetal and neonatal mice following maternal exposure to 8-2 FTOH (Henderson and Smith, 2006). The final chapter incorporates the overall conclusions of this research and how they relate to the objectives of our research. This chapter also discusses

future work that is needed to further expand the state of science of metabolic conversion of 8-2 FTOH in mammals and any resulting toxic effects.

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CHAPTER 2

LITERATURE REVIEW

This literature review will summarize the history and scope of industrial and consumer applications utilizing perfluorinated chemicals as well as the unique physical-chemical properties of PFCs and how these properties dictate analysis. Finally, the pertinent information on the environmental degradation and mammalian metabolism of 8-2 FTOH is reviewed, as well as its toxicology and that of its dominant metabolite PFOA.

Significance of Perfluorinated Chemicals

The global occurrence of perfluorooctanoic acid (PFOA) in environmental and mammalian matrices has spurred regulatory interest in potential sources of this stable compound. PFOA has been shown to be carcinogenic (Biegel et al., 2001) and developmentally toxic (Lau et al., 2004 and Lau et al., 2006) in rodents as well as a stable metabolite formed by both environmental degradation and mammalian metabolism processes. PFOA is non-mobile in the environment and it has been hypothesized that precursor fluorotelomer alcohols (ie. 8-2 FTOH) are, in part, responsible for the concentrations of PFOA being detected globally. 8-2 FTOH, a primary compound used in polymer synthesis, is also found ubiquitously in the environment and can undergo biotic and abiotic transformation to PFOA (Fasano et al., 2006; Hagen et al., 1981; Kudo et al., 2005; Martin et al., 2005).

The fluorotelomer alcohols (FTOHs) are used in the manufacture of fluorinated surfactants and polymers and in the surface-active modification of consumer products (Dinglasan-Panlilio and Mabury, 2006; Ellis et al., 2004; Larsen et al., 2006; Martin et al., 2005; Stock et al., 2004b). FTOHs are linear chain polyfluorinated alcohols in which the –OH moiety links the polyfluorinated alkyl tail to various polymers via ester, amide, urethane, and ether linkages (Dinglasan-Panlilio and Mabury, 2006; Lehmler, 2005; Schultz et al., 2003). Furthermore, the FTOHs are used as intermediates in the synthesis of dyes, paints, adhesives, polymers, and waxes (Dinglasan-Panlilio and Mabury, 2006). The FTOHs have been shown to be present in the North American troposphere at concentrations up to 165 pg/m^3 (Stock et al., 2004b), and it is estimated that the global production from 2000 to 2002 exceeded 5000 tons/year (Prevedouros et al., 2006).

PFCAs are commercial surfactants and used in the manufacture of fluoropolymers while the fluorotelomer alcohols (FTOHs) are used as chemical intermediates in similar polymer synthesis applications (Boulanger et al., 2005; Dinglasan-Panlilio and Mabury, 2006; Kissa, 2001; Prevedouros et al., 2006). Perfluorocarboxylic acids (PFCAs) and their sulfonic analogues, represent an emerging class of persistent organic pollutants due to their resistance to mammalian metabolism (Kudo and Kawashima, 2003; Martin et al., 2005; Martin et al., 2004b; Prevedouros et al., 2006) and minimal environmental biodegradation (Dinglasan et al., 2004; Ellis et al., 2004; Wang et al., 2005a).

The prevalence and resulting global dissemination of these stable compounds remains unexplained and sources for their release as well as subsequent formation from precursor compounds are currently being investigated (Giesy and Kannan, 2001; Kannan et al., 2002a; Kannan et al., 2005; Stock et al., 2004b). Importantly, these compounds have been shown to be

ubiquitous (Giesy and Kannan, 2001; Martin et al., 2002), persistent, bioaccumulative (Martin et al., 2004a; Smithwick et al., 2006; Tomy et al., 2004) and potentially toxic (Butenhoff et al., 2002; Butenhoff et al., 2004a; Kennedy et al., 2004; Lau et al., 2004).

Surfactant Chemistry (Telomerization and Electrochemical Fluorination)

Perfluorinated chemicals (PFCs) are characterized by a fluorine-saturated alkyl chain and a functional polar head group that when coupled with the long-chain perfluorinated alkyl tail makes PFCs immiscible with most liquids, non-flammable, non-corrosive and thermally resistant (Kissa, 2001). As a whole, PFCs are generally hydrophilic, oleophilic and inert (Kissa, 2001). Because of the strength imparted by the carbon-fluorine bond, PFCs are generally resistant to physical, chemical and metabolic degradation (Faithfull and Weers, 1998; Houde et al., 2006; Kissa, 2001; Lehmler, 2005; Prevedouros et al., 2006). Therefore, numerous PFCs are used in both industrial and commercial applications to impart water, heat and acid resistance due to fluorine atom integration (Lehmler, 2005; Mylchreest et al., 2005; Prevedouros et al., 2006).

These compounds have been commercially and industrially produced for over 30 years and are used as stain and water repellents for surface treatment of textiles, carpets, leather, and paper products and as surfactants in fire-fighting foams (Hansen et al., 2002), herbicides and insecticides (Key et al., 1997), lubricants, paints, adhesives and acid etching solutions (Dinglasan-Panlilio and Mabury, 2006; Ellis et al., 2004; Prevedouros et al., 2006; Stock et al., 2004b). Two industrially relevant groups of PFCs are the perfluorinated carboxylic acids (PFCAs) and the polyfluorinated fluorotelomer alcohols (FTOHs).

Fluorotelomer alcohols: FTOHs are polyfluorinated compounds characterized by an even number of perfluorinated carbons and an adjacent methylene group. 8-2 FTOH is one

compound among a class of chemicals that are the primary intermediates used in the preparation of telomer-based polymer products (TBPPs) (Prevedouros et al., 2006). FTOHs have the common structure $CF_3(CF_2)_nCH_2CH_2OH$, where n is an odd number; generally 1 thru 9. The resulting nomenclature refers to the number of fluorine-saturated carbons atoms and unsaturated methylene groups (i.e., 8-2 FTOH: $CF_3(CF_2)_7CH_2CH_2OH$). FTOHs are generally produced by the telomerization of tetrafluoroethylene (taxogen) coupled with a varying number of telogens (perfluoroalkyl iodides) depending on the length of the desired product (Kissa, 2001). The resulting polyfluorinated iodides can then be converted into olefins, fluorotelomer carboxylic acids (FTCAs) or FTOHs to be directly used as fluorosurfactants to reduce the surface tension of viscous emulsions or indirectly as chemical intermediates in similar industrial processes (Lehmler, 2005; Schultz et al., 2003).

Fluorotelomer alcohols are primarily used in polymer formation in which the alcohol moiety bridges the perfluorinated alkyl tail to the polymeric backbone. During, 2000-2002 it was estimated that more than 5×10^6 kg/yr was produced worldwide with greater than 40% of total production in North America (Ellis et al., 2004; Stock et al., 2004b). Of the total FTOHs produced, it is estimated that approximately 80% are used in polymer formation while the remaining 20% is used in the aforementioned processes for its surfactant properties (TRP, 2002).

Production of FTOHs has increased post-2002 to approximately $11\text{-}14 \times 10^6$ kg/yr to accommodate a voluntary PFOS-chemistry phase-out effort (DuPont, 2005). Although the fluorotelomer alcohols are not used directly in industrial and consumer applications, they are used in the synthesis of numerous fluorosurfactants and are incorporated into a large array of polymeric material (Boulanger et al., 2005; Dinglasan-Panlilio and Mabury, 2006; Larsen et al., 2006) including but not limited to, paints, surface-active coatings, polymers, adhesives, waxes,

polishes and electronics (Kissa, 2001). More recently, FTOHs have been utilized to produce commercial products such as cleaning and herbicidal agents (Key et al., 1997; Schultz et al., 2003).

Perfluorinated Carboxylic Acids: In comparison, PFOA and its related carboxylic acids are generally synthesized by electrochemical fluorination of their non-fluorinated counterparts (ie. linear hydrocarbons). Electrochemical fluorination yields are generally on the order of >30% and are limited due to the formation of oxygen difluoride, an explosive byproduct. When carboxylic acid chlorides and fluorides are substituted as starting material, hydrolysis results in the formation of free PFCAs and/or their respective metal salts (Kissa, 2001).

PFOA, resulting from electrochemical fluorination, is generally produced as ammonium perfluorooctanoate (APFO). APFO is then used as a processing aid in the production of various fluoropolymers and in the emulsion polymerization of these polymers. However, this process was discontinued in industrial settings between the years 2000-2002 due to the environmental accumulation of sulfonic analogues (USEPA, 2001). Other preparatory methods of PFCAs include the oxidation of perfluorinated iodides and bromides (Lehmler, 2005).

Environmental Relevance and Sources

Sources for the environmental availability of FTOHs and other precursor compounds include release from industrial and consumer applications (Prevedouros et al., 2006), as residuals in consumer products due to incomplete covalent linkages during polymer production (Dinglasan-Panlilio and Mabury, 2006), and as thermally-mediated polymer degradation products (Dinglasan-Panlilio and Mabury, 2006; Ellis and Mabury, 2003; Yamada et al., 2005). The lifetime of FTOHs in the atmosphere is ≤20 d (Ellis et al., 2003; Wallington et al., 2006) and

they are present in the North American troposphere in pg/m^3 concentrations (Martin et al., 2002; Stock et al., 2004b). FTOH concentrations determined in the North American troposphere range from 17 – 135 pg/m^3 (Martin et al., 2002; Stock et al., 2004b), and it is estimated that a total environmental flux of 5.34 x 10^4 tons/year would be needed to account for current atmospheric concentrations (Prevedouros et al., 2006).

Once released into the environment, these volatile compounds are capable of long-range atmospheric transport while non-volatile PFCs will likely be terrestrially or aquatically deposited at or near the source. PFCAs, due to their low water solubilities and low vapor pressures are non-mobile in the environment (Cobranchi et al., 2006; Krusic et al., 2005; Stock et al., 2004a). The ability to be transported coupled with the atmospheric lifetime of FTOHs, makes these fluorotelomer precursors prime candidates for long-range atmospheric transport and subsequent metabolism, degradation and transformation processes resulting in the formation of PFOA and other stable PFCAs in areas far removed from their production or use. Thus, PFCAs have become ubiquitous in the environment (Giesy and Kannan, 2001; Kannan et al., 2002a; Skutlarek et al., 2006; Taniyasu et al., 2003; Yamashita et al., 2005). PFCA homologues have been detected in fish tissue following AFFF release into a watershed (Moody et al., 2002), determined in biota samples from the Canadian Artic (Martin et al., 2004b) and present in human blood (Alexander et al., 2003; Butenhoff et al., 2004b; Calafat et al., 2006a; De Silva and Mabury, 2006; Harada et al., 2006; Karrman et al., 2006; Olsen et al., 2003b).

Environmental sources of FTOHs are currently unknown but it is hypothesized that these chemicals may be released from both industrial applications as well as the decomposition of polymeric or nonpolymeric materials that use FTOHs during manufacture. These chemicals have also been shown to be present as residuals in commercially available end-products (Ellis et

al., 2003). Residual fluorotelomer alcohols would result from incomplete covalent linkages or incomplete product purification prior to commercial use of polymeric products that rely on FTOHs as chemical intermediates (Dinglasan-Panlilio and Mabury, 2006; Larsen et al., 2006). Of seven industrially-applied or consumer-used products, all contained free or unbound FTOHs, with chains ranging from 8 to 14 carbons (Dinglasan-Panlilio and Mabury, 2006).

Both sources, degradation products and residuals in consumer products, illustrate how FTOHs may end up in wastewater treatment facilities in which they are subjected to both aerobic and anaerobic degradation by microbes (Dinglasan et al., 2004; Wang et al., 2005a; Wang et al., 2005b). Furthermore, their presence in the North American atmosphere suggests that FTOHs are subject to atmospheric oxidation as well as mammalian and non-mammalian metabolism following exposure (Stock et al., 2004b).

Since PFOA is used in the manufacture of fluoropolymers, it has been postulated that direct industrial releases coupled with the environmental metabolism and biodegradation of precursor compounds (i.e. 8-2 FTOH) results in the concentration of PFCAs detected in both environmental and mammalian matrices worldwide (Stock et al., 2004b). However, possible release and exposure routes are still being elucidated. Sources for the release of PFOA are similar to those of 8-2 FTOH and include the thermal breakdown of perfluorinated polymers in cookware and internal combustion engines (Yamada et al., 2005), losses from production and application facilities (Prevedouros et al., 2006), and losses from treated consumer articles (Begley et al., 2005; Prevedouros et al., 2006; Washburn et al., 2005)

Neither of these source terms, however, seems likely to account for the global distribution of PFOA currently being reported. Some have hypothesized that the volatile perfluoroalkyl telomers released during preparation of telomer-based products, are readily

transported via atmospheric processes and subsequently transformed into PFOA (De Silva and Mabury, 2004; Dinglasan-Panlilio and Mabury, 2006; Martin et al., 2005; Smithwick et al., 2005). Still others have posited that PFOA released from landfills and sewage treatment facilities has migrated to the oceans where it became entrained in marine aerosols and, subsequently, rained out upon the land (Prevedouros et al., 2006). Furthermore, sources of PFOA include the incorporation of water-soluble but non-volatile salts such as ammonium perfluorooctanoate in the processing of fluoropolymers and their subsequent entry into aquatic ecosystems (Kissa, 2001).

Analytical Chemistry of Perfluorinated Chemicals

To detect PFCs in environmental and mammalian matrices, the majority of current analytical techniques rely on liquid chromatography, tandem mass spectrometry LC-MS/MS for the detection and quantification of perfluorocarboxylic acids (PFCAs) and perfluorooctane sulfonate (PFOS) and gas chromatography, mass spectrometry (GC/MS) for volatile PFCs. Appropriate analytical methodology, sampling, sample preparation procedures, as well as applicable measurement techniques are being established by many researchers for PFCs that are amenable to analysis by LC-MS/MS.

LC–MS/MS is the predominant method utilized for the low-level, compound-specific quantification of PFCAs (Berger et al., 2004; Flaherty et al., 2005; Karrman et al., 2005; Larsen et al., 2006; Maestri et al., 2006; Ohya et al., 1998; Reagen et al., 2004; Stadalius et al., 2006; Szostek et al., 2006) while gas chromatography, mass spectrometry (GC/MS) methodology has been traditionally utilized for determination of FTOHs or derivatized acids (Belisle and Hagen, 1980; Dinglasan et al., 2004; Ellis et al., 2004; Henderson et al., 2006; Kudo et al., 1998; Larsen

et al., 2006; Martin et al., 2002; Reagen et al., 2004; Szostek and Prickett, 2004; Yang et al., 2002; Ylinen et al., 1985). Although GC(NCI)/MS has proven useful for the detection of volatile fluorotelomer-based chemicals, resolution of intermediate and terminal acids resulted in significantly greater detection limits (Ellis and Mabury, 2003). Because of the inter-relationship among these compounds, particularly in small biological samples, there is a great need to measure both types of analytes by a single technique.

Appropriate analytical methodology, sampling, sample preparation procedures, as well as applicable measurement techniques are being established by many researchers for PFCs that are amenable to analysis by LC–MS/MS, most notably PFOA. However, use of LC–MS/MS is often problematic due to instrumental components that contain many of these fluorotelomer-based chemicals as well as C6-C10 PFCAs. A few publications have reported the analysis and quantification of fluorotelomer alcohols (FTOHs) in mammalian matrices but few allow for the simultaneous detection of volatile and non-volatile compounds. An analysis of the 8-2 FTOH in biological matrices (rat serum, liver and kidney tissue) has been established, but did not focus on the concurrent analysis of both 8-2 FTOH and PFOA (Flaherty et al., 2005; Szostek and Prickett, 2004).

There is a great need for reliable analytical methodologies to facilitate both environmental monitoring and toxicological studies of the perfluorinated compounds as well as 8-2 FTOH and its metabolites (Martin et al., 2004b). Furthermore, there is a great need for method advancement and analytical techniques that allow the quantification of low levels of residual fluorotelomer raw materials and reaction by-products in environmental and product formulations (Begley et al., 2005; Dinglasan-Panlilio and Mabury, 2006; Schultz et al., 2004; Washburn et al., 2005). The analytical methodology for these neutral or anionic compounds is

complicated by their unique physicochemical properties, the lack of commercially available standards, instrumental and matrix background contamination and complex mixtures detected worldwide. Currently, ion-pair or solid phase extraction techniques are employed followed by LC-MS/MS analysis of non-volatile metabolites and GC/MS analysis of volatile compounds (Hansen et al., 2001). Overall, comparing data across laboratories is hampered by the above mentioned analytical problems, and analytical improvements are urgently needed (Berger and Haukas, 2005; Berger et al., 2004).

Initial analytical techniques relied on combustion (Belisle and Hagen, 1980; Hagen et al., 1981) to determine total fluoride in sample; however, since PFCs are resistant to thermal degradation, these early techniques often underestimated the fluorine content in the sample. Several derivatives of early techniques have been employed such as combination with ion-selective electrode detection with little success. NMR then became a preferred method from 1960-2004 to identify the presence of CF_2 and CF_3 groups in environmental samples but even when coupled with LC-MS/MS, mis-quantification can occur (Martin et al., 2002). Recently, more reliable analysis incorporating liquid and/or gas chromatography are employed.

Martin et al. (2002) first reported a method for the determination of FTOHs in environmental samples using GC/MS (both negative chemical ionization (NCI) and positive chemical ionization (PCI)). The use of chromatography for both volatile and non-volatile PFCs began when Martin and co-workers (2002) developed a GC(CI)/MS method for the detection of several volatile compounds in air samples. The derivatization of and subsequent analysis of non-volatile PFCs, coupled with GC/flame ionization detector (FID) and GC/electron capture detector (ECD) was illustrated by various researchers (Belisle and Hagen, 1980; Berger et al.,

2004; Moody et al., 2001; Sottani and Minoia, 2002; Ylinen et al., 1985). However, LC-electrospray ionization (ESI)/MS dominated over latter techniques.

In recent years, many articles using liquid chromatographic analysis of PFCs have been published (Calafat et al., 2006a; Calafat et al., 2006b; serum and breast milk); (Moody et al., 2003; surface waters); (Taniyasu et al., 2003; water, fish, birds and humans from Japan). Liquid chromatography was used, coupled to various detectors, to monitor PFCs in water (Hori et al., 2005) and biological samples (Ohya et al., 1998). In comparing different LC-MS based detection and quantification methods including ion-trap MS, time-of-flight (ToF)/MS, and triple quadrupole MS, Berger and co-workers selected perfluorohexanoic acid (PFHxA), PFOA, PFOS, PFOSA and 4-2 FTOH, 6-2 FTOH, 8-2 FTOH as model compounds (Berger et al., 2004).

However, there are few published analytical methods for determination of fluorotelomer alcohols in complex biological or environmental matrices in current literature. Szostek and Prickett (2004) utilized EI for determination of 8-2 FTOH in rat plasma, liver, kidney and adipose tissue with limits of detection (LODs) ranging from 4-12 ng/g. Berger et al., (2005) developed a LC/MS based method for the analysis and quantification of 4-2, 6-2 and 8-2 FTOHs and then compared the sensitivity of LC/MS methods and published GC/MS methodology (Berger and Haukas, 2005). Selected ion monitoring (SIM) allowed for an increase in sensitivity as well as a decrease in interferences resulting in a 0.7 ug/g detection limit for both PFOA and 8-2-8 ester. Furthermore, LC-MS/MS analysis of FTOHs and FTCAs in water and biota allowed for 100 pg/L method detection limit (MDL) for water and 100 ppt (pg/g) levels in biological matrices (Taniyasu et al., 2005; Yamashita et al., 2004).

Environmental and Mammalian Transformation of 8-2 FTOH: Formation of PFOA

Recent studies have shown that PFOA is an end product of the microbially mediated oxidation of 8-2 FTOH in a mixed-microbial system and activated sludge (Dinglasan et al., 2004; Wang et al., 2005a). The β-oxidation of 8-2 FTOH, resulting in the formation of PFOA, has also been shown to occur in the mammalian liver via liver peroxisomes for which the reactions mimic that of fatty-acid oxidation (Kudo et al., 2005). Furthermore, 8-2 FTOH has been shown to be the metabolized *in vitro* into PFOA by both rat and human hepatocytes (Martin et al., 2005). This β-oxidation pathway is proposed to occur in aerobic soils and sediments, as well due the metabolite profiles determined in abiotic transformation experiments (Dinglasan et al., 2004; Wang et al., 2005a). Non-volatile intermediates of 8-2 FTOH metabolism include 8-2 fluorotelomer carboxylic acid (8-2 FTCA), 8-2 fluorotelomer unsaturated carboxylic acid (8-2 FTUCA), 8-2 fluorotelomer aldehyde (8-2 FTAL), and 8-2 fluorotelomer unsaturated aldehyde (8-2 FTUAL), and the non-volatile terminal metabolites are perfluorononanoic acid (PFNA) and PFOA resulting from α- and β-oxidation, respectively. Notably, no evidence has been found for subsequent metabolic or environmental biodegradation of the end metabolites, PFOA or PFNA (Fasano et al., 2006; Martin et al., 2005).

Environmental Degradation of 8-2 FTOH

The environmental degradation of 8-2 FTOH has been studied in the atmosphere (Ellis et al., 2004), mixed-microbial cultures (Dinglasan et al., 2004) and activated sludge (Wang et al., 2005b). Laboratory studies, mimicking natural reactions thought to be occurring in the atmosphere, have shown that various PFCAs are formed during the oxidation of fluorotelomer alcohols (FTOHs) (Ellis et al., 2004; Sulbaek Andersen et al., 2005; Wallington et al., 2006) and

FTOHs have atmospheric lifetimes of approximately 20 days (Ellis et al., 2003). In the atmosphere, FTOHs are subjected to oxidation reactions initiated by the presence of OH radicals (Ellis et al., 2003). However, when OH radicals were replaced with Cl atoms, each FTOH tested (4-2, 6-2 and 8-2 FTOH) resulted in an intact perfluoroalkyl chain as determined by LC-MS/MS analysis (Ellis et al., 2004). During atmospheric oxidation of 8-2 FTOH, PFOA and PFNA accounted for approximately 3.1% of oxidation products. Approximate molar yield of PFOA and PFNA (cumulative) formed by the atmospheric oxidation of 8-2 FTOH is ≤12% (Wallington et al., 2006).

In aerobic conditions, 8-2 FTOH degradation and subsequent formation of the terminal acids, PFOA and PFNA occurs. When exposed to a mixed microbial system (aerobically) or municipal wastewater treatment sludge (Lange, 2002) both even and odd chained PFCAs are produced suggesting d proposing that both β- and α- oxidation pathways are occurring. FTUCAs of the corresponding FTOHs were all detected as transient intermediates in a 16 day incubation study (Dinglasan et al., 2004). It was concluded that 8-2 FTOH can be oxidized by an alcohol dehydrogenase enzyme in bacteria to 8-2 FTAL and further oxidation of the terminal carbon leads to the formation of the 8-2 FTCA. Finally, a subsequent round of β-oxidation of 8-2 FTCA results in the formation of PFOA. However, whether or not α-oxidation occurs in activated sludge is controversial. In a similar test system, 7-2 FTOH, 7-3 FTUCA, 7-3 FTUAmide along with 8-2 FTAL, 8-2 FTCA, 8-2 FTUCA, 7-3 FTCA, and PFOA were observed (Wang et al., 2005b). The absence of PFNA however, suggests that α-oxidation processes are absent (Wang et al., 2005b).

Mammalian Metabolism of 8-2 FTOH

Hagen et al. (1981) first identified probable mammalian metabolism of 8-2 FTOH. 8-2 FTCA and PFOA were identified as metabolites in rats by 19F NMR gas chromatography coupled to a microwave plasma detector (Hagen et al., 1981). Furthermore, based on retention time matching with GC/ECD, it was hypothesized that 8-2 FTUCA is also an intermediate metabolite. This work was expanded by both Martin et al. (2005) and Kudo et al. (2004) in which metabolites were tracked *in vitro* and *in vivo*, respectively. *In vitro*, 8-2 FTCA and 8-2 FTUCA were formed rapidly and detected in blood, liver and kidney as early at 6 hours post-treatment. Further data validation was conducted by incubating hepatocytes with 8-2 FTUCA or 8-2 FTUCA which resulted in the formation of 8-2 FTUCA, PFOA and PFNA and only PFOA, respectively. Other novel metabolites included 7-2 FTCA and 7-2 FTUCA (Martin et al., 2005).

Recently, the adsorption, distribution, metabolism and excretion of 8-2 FTOH was determined in rats (Fasano et al., 2006). Following a single oral dose of 8-2 FTOH (5 and 125 mg/kg), 8-2 FTOH was at maximal concentrations at or before 1 hour post-treatment and cleared rapidly with a half-life of approximately 5 hours. 8-2 FTCA was the next most abundant metabolite and had a comparable half-life of 4-18 hours, similar between sexes. However, the $t_{1/2}$ of PFOA was longer in males (112-217 h) when compared to females (5.6-17.5 h). The majority (>70%) of 8-2 FTOH was excreted in feces exclusive of sex and dose level, 4% in urine of which approximately 1% was determined to be PFOA. Bile metabolites include both glucuronide and glutathione conjugates, and PFHxA. Metabolites identified included: PFHxA, PFHA, PFOA, PFNA, 7-3 FTUCA, 7-3 FTCA, 8-2 FTUCA, 8-2 FTCA, as well as cysteine, glucuronide, N-acetyl cysteine, cysteinylglycine, sulfatase (*in vitro* only) and glutathione conjugates (Fasano et

al., 2006). In the above study, approximately 2-fold more total radioactivity was determined in female compared to male rat urine (Fasano et al., 2006).

While gender differences are seen in rats, this sex-mediated route of elimination is absent in most other species (Houde et al., 2005; Kannan et al., 2002b; Smithwick et al., 2005). Rats possess an organic acid transport mediated excretion process that rapidly and efficiently decreases the body burden of PFOA (Hundley et al., 2006; Kudo et al., 2002), most notably in female rats and post-pubescent pups (Hinderliter et al., 2006). Humans are believed to be absent this anion excretion mechanism as well as gender-mediated excretion mechanisms of PFCs (Butenhoff et al., 2004b) and therefore rats are not an appropriate animal model for elucidating the human health risk of PFOA exposure.

Mammalian metabolism as seen in Hagen et al. (1981) and in Martin et al. (2005) suggest that exposure to precursor compounds may help account for the PFCAs determined in human blood samples (Ehresman et al., 2006; Emmett et al., 2006; Olsen et al., 2003a; Olsen et al., 2004; Olsen et al., 2003b). Furthermore, isomeric profiles seen in polar bears (Langlois and Oehme, 2006) suggest that the concentration of straight-chain perfluorinated carboxylic acids detected originate from straight-chained precursor compounds such as the FTOHs (Martin et al., 2004b). The most plausible explanation for global dissemination is that terminal acids are formed as metabolism products (Kudo et al., 2005; Martin et al., 2005) or as degradation products of several structurally-related homologues in the atmosphere coupled with processes detailing β-oxidation under aerobic environmental conditions (Dinglasan et al., 2004; Wang et al., 2005a).

Toxicology of 8-2 FTOH and the Metabolites, PFOA and PFNA

Information on the toxicological effects of 8-2 FTOH is limited. As a class, PFCs have been shown to cause weight loss, hepatocellular hypertrophy, reduction of serum cholesterol, adverse effects on thyroid hormone levels, and neonatal mortality (Butenhoff et al., 2002; Butenhoff et al., 2004a; Kennedy et al., 2004; Lau et al., 2004; Luebker et al., 2002; Perkins et al., 2004; Seacat et al., 2002; Seacat et al., 2003). Perfluorinated compounds have also been shown to cause alterations in fatty acid composition of both serum and hepatic lipids (Iwase et al., 2006; Kudo et al., 2005). Mammalian toxicity studies for PFOA have been conducted in monkeys (Butenhoff et al., 2002), rabbits, mice, rats, and other species (Hundley et al., 2006). Although these studies show that pharmacokinetics of the fluorinated compounds vary between species, PFOA has been shown to bioaccumulate in higher trophic organisms including humans (Olsen et al., 2005; Tomy et al., 2004) with a teratogenic potential (Lau et al., 2004; Lau et al., 2006).

Subchronic and developmental toxicity of 8-2 FTOH as well as a telomer alcohol mixture has been investigated (Mylchreest et al., 2005). In a 90-day subchronic study, the NOAEL for 8-2 FTOH was 5 and 125 mg/kg/day for male and female rats, respectively. Increases in peroxisome proliferation were also noted between 25 and 125 mg/kd/day for both sexes. Treatment with 8-2 FTOH did not lower body weight nor did it affect the overall health of the animals. Relative liver weights were increased in a dose-dependent fashion (Mylchreest et al., 2005b). In rats, chronic oral exposure to 8-2 FTOH increased oleic acid and arachidonic acid while linoleic acid, palmitic acid, eicosapentaenoic acid and docosahexaenoic acid were all decreased to varying extents. Palmitoyl-CoA chain elongase was significantly increased relative to dose as was the activity of stearoyl-CoA desaturase (Iwase et al., 2006).

The developmental toxicity of 8-2 FTOH was investigated in the rat. Timed-pregnant Crl:CD rats were orally exposed to either 0, 50, 200, or 500 mg/kg from gestational day (GD) 6-20 and maternal toxicity was seen only at the highest dose group and manifested as mortality, decreased body weight gains (beginning on GD7), reduced food consumption and obvious clinical signs of morbidity (Mylchreest et al., 2005b). At 500 mg/kg/d, developmental toxicity included increased fetal skeletal variations such as delayed fetal skull bone ossification. The NOAEL was defined as 200 mg/kg/day and therefore 8-2 FTOH is not considered a developmental toxicant in the rat due to the magnitude of dose required to elicit negative developmental responses (Mylchreest et al., 2005b).

The toxicological impacts of 8-2 FTOH exposure have been shown to mimic those induced by PFOA likely due to the rapid metabolism of 8-2 FTOH to PFOA. Kudo et al. (2005) chronically fed male ddY mice 8-2 FTOH (0.025, 0.05, 0.1, and 0.2% w/w) for 7, 14, 21, and 28 days. 8-2 FTOH resulted in liver enlargement as well as increases in relative liver weight in a dose and time-dependent manner via a hypothesized mechanism of peroxisome proliterative activity. PFOA was confirmed as a metabolite and accumulated in the liver over the course of the study. They suggest that PFOA concentration, due to its linear relationship with peroxisomal acyl-CoA oxidase activity, was responsible for the toxic effect. The higher the dose 8-2 FTOH and corresponding increased concentration of PFOA, the shorter the time required for reaching maximum activity of the enzyme. The number as well as the volume density (percentage of cytoplasmic area) of peroxisomes was increased in hepatocytes. Furthermore, 8-2 FTOH disrupts the endocrine system by behaving like xenoestrogens and inducing cell proliferation (Maras et al., 2006). It was concluded that FTOH exposure results in PFOA-like *in vivo* effects (Maras et al., 2006).

The toxicological information pertaining to PFCAs is largely limited to PFOA and perfluorodecanoate, however, PFNA and PFuDA produce effects that are similar to those elicited by PFOA and PFDA (Goecke-Flora and Reo, 1996; Kudo et al., 2000). The toxicology of PFCAs range from the inhibition of gap junction intercellular communication (Hu et al., 2002) to hepatic tumor promotion (Biegel et al., 2001) and peroxisome proliferation (Berthiaume and Wallace, 2002).

PFOA has carcinogenic (Biegel et al., 2001) effects due to its actions as a peroxisome proliferator as well as general developmental toxicity (Lau et al., 2004). This evidence has prompted regulatory action to determine the potential human-health impact of perfluorinated chemicals. PFCAs have been found to adversely affect both pre- and post-natal development (Case et al., 2001; Lau et al., 2004; Thibodeaux et al., 2003). However, PFOA is a known developmental toxicant. The developmental toxicity of PFOA and perfluoroalkyl derivatives has been reviewed by Butenhoff et al.(2004) and the general toxicity reviewed by Kennedy et al. (2002).

PFOS is developmentally toxic in rats (Butenhoff et al, 2004a; Grasty et. al., 2005, Thibodeaux et al., 2003) and PFOA is developmentally toxic in mice (Lau et al., 2004, Lau et al., 2006) resulting in embryonic and post-natal death and growth retardation when animals are administered at least 10 mg PFOS or PFOA/kg/day over total gestation. Interestingly, it is suggested that female rats, unlike humans, rapidly excrete PFOA (Kudo et al., 2002, Kudo et al., 2001). It is hypothesized that rats possess an organic acid transport (OAT)-mediated excretion process that rapidly and efficiently decreases the body burden of PFOA (Kudo et al., 2002), as well as other perfluorinated chemicals. These processes are not maximally expressed until sexual maturity in rats (Buist et al., 2002, Hinderliter et al., 2006).

Although the OAT transporters are highly conserved across species it has been shown that modulation and transcription of various isoforms (namely OAT2) are age- and sex-dependent in rats but less so in mice (Buist et al. 2002, Buist and Klaasen, 2004). In comparison, in the human kidney, OAT2 is the most abundant transcript detected (Bahn et al., 2004); however, gender-based differences in humans have not been definitively elucidated. Based on available data, male (Buist et al., 2002; Hinderliter et al., 2006, Kudo et al., 2002) and female (Lau et al., 2003) rats do not appear to be appropriate animal models for understanding the human health risk of PFOA exposure (Harada et al., 2005, Calafat et al., 2006).

More recently, the effects of PFOA exposure during pregnancy were investigated in the mouse. PFOA produced dose-dependent effects on the number of resorptions, maternal and fetal weight gain, and post-natal survival and growth deficits at doses between 10-30 mg PFOA/kg/day with full litter loss occurring at 40 mg PFOA/kg/day (Lau et al., 2006). Early gestational exposure has been associated with growth reduction, low body weight, and poor post-natal survival of pups (Lau et al., 2006). Lau et al. (2006) concluded that *in utero* exposure to PFOA provides the major contribution of the associated adverse effects and lactational exposure may be a minor contributor to developmental outcomes. Furthermore, neonatal PFOA exposure results in developmental neurotoxic effects (Johansson et al., 2006). Both PFOA and PFDA adversely affect the cholinergic system in adult mice after neonatal exposure as evidenced by deficits in locomotion and total activity in adult mice, similar to other persistent organic pollutants (Johansson et al., 2006).

In rats, Hinderliter et al. (2005) observed that following gestational exposure, PFOA milk concentrations were proportional to the maternal administered dose and thus neonates received a constant dose from the dam until weaning as a result of lactational transport. Secondly, upon

repeated dosing with PFOA, approximately 8% of the total administered maternal dose is transferred to the developing neonate via lactation (Hinderliter et al., 2005).

In conclusion, information on the metabolism of 8-2 FTOH in mice is limited. 8-2 FTOH has been shown to be metabolized into PFOA both *in vivo* and *in vitro*. However, there are few studies examining the metabolism in mice and no studies which focus on maternal-fetal and neonatal distribution of 8-2 FTOH and its oxidation metabolites, PFOA and PFNA. The adsorption, distribution and metabolism of 8-2 FTOH has been investigated in the rat (Fasano et al, 2006), as well as the developmental toxicity during pregnancy (Mylchreest et al., 2005b). In rats, 8-2 FTOH increases fetal skeletal variations at 500 mg/kg/d with a no observed adverse effect level defined as 200 mg/kg/d (Mylchreest et al., 2005) and behaves like xenoestrogens *in vitro* (Maras et al., 2006). However, since rats appear to be an inappropriate animal model, more research is needed to fully understand the human-health risks associated with precursor (8-2 FTOH) exposure.

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CHAPTER 3

QUANTIFICATION OF FLUOROTELOMER-BASED CHEMICALS IN MAMMALIAN MATRICES BY MONITORING PERFLUOROALKYL CHAIN FRAGMENTS WITH GC/MS[1]

[1] Henderson, W. Matthew, Eric J. Weber, Stephen E. Duirk, John W. Washington, and Mary Alice Smith. 2006. *J Chromatogr B Analyt Technol Biomed Life Sci. Available online 26 September 2006, 10.1016/j.jchromb.2006.08.042.*

Abstract

Perfluorocarboxylic acids (PFCAs), namely perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA), have been identified as persistent, bioaccumulative and potentially toxic compounds. The structural analog, 8-2 fluorotelomer alcohol (8-2 FTOH) is considered the probable precursor of these stable metabolites. Because simultaneous quantification is needed for volatile and non-volatile perfluorinated chemicals (PFCs) in complex matrices, a GC/MS method was developed and tested based on selected ion monitoring of perfluorinated alkyl parent chain fragment ions. Although the method requires a derivatization step, combined GC/MS analysis of PFCA-me's and FTOHs increases analytical efficiency and decreases sample analysis time. The method instrument detection limits are between 7.1 and 24.5 ng/mL extract (MTBE), and the method quantification limits are below 50 ng/mL serum or ng/g liver for all PFCs investigated. Recoveries from mouse serum and liver homogenates, which were spiked with FTOHs and PFCAs at levels of 25 and 200 ng/mL or ng/g, ranged from 81 to 101%. Finally, the utility of the method was demonstrated by dosing male CD-1 mice with 30 mg/kg-BW of 8-2 FTOH and quantifying PFCs 6 h post-treatment. The advantages of this method are (1) the simultaneous detection of both volatile and non-volatile fluorotelomer-based chemicals in complex matrices such as mammalian tissues, (2) as a confirmatory method to LC-MS/MS, and (3) as an alternative method of analysis for laboratories without access to LC-MS/MS.

Introduction

The concern over perfluorinated chemicals (PFCs) has been propagated by their widespread use in consumer products coupled with their persistence in the environment (Dinglasan-Panlilio and Mabury 2006). Of particular concern is the environmental and mammalian bioavailability of perfluorooctanoic acid (PFOA) which has been shown to be globally distributed (Giesy et al. 2001), bioaccumulative (Kannan et al. 2005; Martin et al. 2004), persistent and potentially toxic (Kennedy et al. 2004; Lau et al. 2004).

Sources for the release of PFOA into the environment include the thermal breakdown of perfluorinated polymers in cookware and internal combustion engines, losses from production and application facilities (Prevedouros 2006), and losses from treated consumer articles (Dinglasan-Panlilio and Mabury 2006; Stock et al. 2004). Neither of these source terms, however, seems likely to account for the global distribution of PFOA currently being reported. Some have hypothesized that the volatile perfluoroalkyl telomers released during preparation of telomer-based products, are readily transported via atmospheric processes and subsequently transformed into PFOA (Ellis et al. 2004; Stock et al. 2004). Of interest among the polyfluorinated telomers are the fluorotelomer alcohols (FTOH), in particular 8-2 FTOH. The FTOHs have been shown to be present in the North American troposphere at concentrations ranging from 11 to 165 pg/m^3 (Stock et al. 2004), and it is estimated that the global production in 2002 exceeded 6.5 million kg/yr (Prevedouros 2006).

The β-oxidation of 8-2 FTOH, resulting in the formation of PFOA, has been shown to occur in the mammalian liver via liver peroxisomes for which the reactions mimic that of fatty-acid oxidation (Kudo et al. 2000; Kudo et al. 2005). 8-2 FTOH has been shown to be the metabolized *in vitro* into PFOA by both rat and human hepatocytes (Dinglasan et al. 2004;

Martin et al. 2005). Notably, no evidence has been found for subsequent metabolism of the terminal acids, PFOA or PFNA (Dinglasan et al. 2004; Fasano et al. 2006; Kudo et al. 2005; Martin et al. 2005; Wang et al. 2005).

Liquid chromatography, tandem mass spectrometry (LC-MS/MS) is the predominant method utilized for the low-level, compound-specific quantification of PFCAs (Flaherty et al. 2005; Powley et al. 2005; Sottani and Minoia 2002) while gas chromatography, mass spectrometry (GC/MS) methodology has been traditionally utilized for determination of FTOHs (Martin et al. 2002; Szostek and Prickett 2004). Although GC(NCI)/MS has proven useful for the detection of volatile fluorotelomer-based chemicals, resolution of intermediate and terminal acids resulted in significantly greater detection limits (Ellis and Mabury 2003; Martin et al. 2002). Because of the inter-relationship among these compounds, there is a great need to measure both of these types of analytes by a single technique, particularly in small biological samples (Hansen et al. 2001).

Appropriate analytical methodology, sampling, sample preparation procedures, as well as applicable measurement techniques are being established by many researchers for PFCs that are amenable to analysis by LC-MS/MS, most notably PFOA (Flaherty et al. 2005; Stadalius et al. 2006). However, use of LC-MS/MS is often problematic due to instrumental components that contain many of these fluorotelomer-based chemicals as well as their metabolic products. A few publications have reported the analysis and quantification of fluorotelomer alcohols (FTOHs) in mammalian matrices with detection limits comparable to this study (Martin et al. 2005; Szostek and Prickett 2004). An analysis of the 8-2 FTOH in biological matrices (rat serum, liver and kidney tissue) has been established, but did not focus on the simultaneous analysis of both volatile and non-volatile metabolites (Szostek and Prickett 2004).

We report a method for the simultaneous analysis of C6-C12 PFCs (FTOHs and methyl esters of PFCAs) in both serum and liver. Following ion-pair extraction of PFCAs and derivatization with diazomethane, extracts that also contain the FTOHs were analyzed with GC/MS using selected ion monitoring (SIM) of the perfluoroalkyl parent chain fragments produced by electron ionization (EI). The mass fragments produced by the FTOHs and PFCA-me's are unlikely to be inherently present in biological samples as evidenced by blank matrix analysis of both serum and liver. Monitoring the ratios of three fragment ions determined in standards coupled with retention times of the standards allows PFC analysis and quantification.

Experimental Materials and Methods

Chemicals: All chemical reagents used in this study were obtained at the highest purity, greater than 98% as determined by the supplier. Perfluorooctanoic acid (PFOA; CAS Number 335-67-1), perfluorononanoic acid (PFNA; CAS Number 375-95-1), 8-2 fluorotelomer alcohol (8-2 FTOH; CAS Number 678-39-7), methyl perfluorooctanoate (PFOA-me; CAS Number 376-27-2), methyl perfluorononanoate (PFNA-me; CAS Number 51502-45-5), methyl perfluoroheptanoate (PFHA-me; CAS Number 14312-89-1), and 3-(perfluorooctyl) propanol (3-PFOP; CAS Number 1651-41-8), were purchased from Oakwood Research Chemicals (West Columbia, SC) as were all other PFCs used for comparative analysis. Both 8-2 fluorotelomer acid (8-2 FTCA; CAS Number 27854-31-5) and 8-2 fluorotelomer unsaturated acid (8-2 FTUCA; CAS Number 70887-84-2) were purchased from TerraChem, Incorporated (Shawnee Mission, KS). Tetrabutylammonium hydrogen sulfate (TBAHS) and sodium carbonate, were purchased from Aldrich Chemical (Milwaukee WI), as were Diazald®, carbitol, and potassium hydroxide.

Mammalian Matrices: Male CD-1 mice were purchased from Charles River Laboratory (Raleigh, NC). Before initiating the study, animals were allowed to acclimate for 7 d in micro-isolator cages (5 animals per cage). The cages were housed in temperature and humidity controlled rooms maintained at 21 to 26 ºC and 30 to 70% RH with a 12 h light/dark cycle. Animals were fed Lab Diet Certified Rodent Chow with both food and water provided *ad libitum*. Mice were gavaged with 8-2 FTOH in a 1:1 mixture of deionized water: propylene glycol at a dose ratio of 30 mg/kg body weight (BW). Animals were sacrificed 6 h post-treatment by carbon-dioxide asphyxiation and blood and liver samples were collected for PFC analysis. Tissues for spike-recovery experiments were similarly obtained from animals receiving vehicle controls. All tissues were flash frozen with a combination of liquid nitrogen and dry ice, and stored at -80 ºC until analyses were complete.

Calibration Standard Preparation and Matrix Spiking: Concentrated solutions (5 and 40 µg/mL) of each standard were prepared in MTBE and diluted as required. Recovery experiments in thawed mammalian matrices were conducted by spiking 1 mL of serum or 1 g of liver with 5 µL concentrated standard solutions in methanol to achieve final PFC levels of 25 or 200 ng/mL or ng/g, respectively.

Sample Extraction and Derivatization: With slight modifications, PFCAs and FTCAs in serum and liver homogenates were extracted using a previously published ion-pair extraction method (Hansen et al. 2001). The method enabled concurrent liquid-liquid extraction of the FTOHs and PFCAs. Briefly, 1 mL of serum was added to 1 mL of 0.5 M TBAHS solution (adjusted to pH 10 with sodium hydroxide), 2 mL of 0.25 M sodium carbonate buffer and 10 µL of internal standard (20 µg/mL PFHA-me and 100 µg/mL 3-PFOP) in a 15 mL polypropylene tube. After thorough mixing, 2 mL MTBE was added to the solution and the solution vortexed

for 15 min. The mixture was centrifuged for 10 min and 1.75 mL of the organic layer was removed for GC/MS analysis of PFCs. Sequential MTBE washes did not dramatically increase analyte recovery and concentration of the MTBE extracts under nitrogen flow resulted in significant 8-2 FTOH losses (>40%). Because these steps did not improve extraction efficiency and method performance, additional MTBE washes and concentration steps were not included in the final methods (data not shown). The derivatization of non-volatile metabolites (PFCAs and FTCAs) was performed by reacting the carboxylic acids with diazomethane to form the more volatile methyl esters. Diazomethane was generated with a Wheaton micro-generator (Aldrich Chemical Company, Milwaukee, WI) by reacting Diazald® with potassium hydroxide (KOH) as described by Ngan and Toofan (Ngan and Toofan 1991). Samples were derivatized by adding 0.75 mL of MTBE from the extraction step to 0.25 mL of ether-trapped diazomethane for 30 min.

Similarly, liver extractions were also performed using the combined liquid-liquid and ion-pair extraction technique. For the extraction of liver samples, a liver homogenate of 1.0 g of liver diluted in 1.0 mL of Milli-Q water was prepared using an electric homogenizer. The homogenate was transferred to a new polypropylene tube containing the same reagents as the serum extraction (discussed above).

GS/MS Conditions: Samples were analyzed using a HP 6890 Series Gas Chromatograph equipped with a 5973 Mass Selective Detector and a HP 6890 Series Injector (Palo Alto, CA). The GC oven was equipped with a Restek (Bellefonte, PA) RTX200MS (30 m x 0.25 mm ID, 0.5 μm df) trifluoropropylmethyl-polysiloxane-phase column. Samples were introduced (2 μL) into the capillary inlet operated in splitless mode (injector temperature 225 ^{o}C). The oven temperature was held for 1 min at 60 ^{o}C, ramped 5 ^{o}C/min to 150 ^{o}C, increased 50 ^{o}C/min to 250

^{o}C and then held for 4 min (total run time equals 25 min) under constant pressure (1.02 psi) with an approximate flow of 0.5 mL/min. The mass spectrometer was operated in selected ion monitoring (SIM) mode with scanning at *m/z* values 131, 169, 231, and 331 with a dwell time of 100 msec. The carrier gas was helium and the transfer line maintained at 280ºC. Furthermore, the MS quad and source were set at 150 ^{o}C and 230 ^{o}C, respectively, with an electron energy of -70 ev and 1294 EM volts. All silicone and Teflon® based analytical products were avoided during sample preparation and GC/MS analysis.

Results and Discussion

The majority of current analytical techniques rely on LC-MS/MS for the detection and quantification perfluorocarboxylic acids (PFCAs) and GC-MS for the detection of volatile, precursor compounds. Our effort has focused on the development of analytical methodology for telomer alcohols (namely 8-2 FTOH), as well as the metabolic products of 8-2 FTOH, predominately PFOA and PFNA. In addition, the analytical method allows monitoring of the intermediate, non-volatile FTCAs, 8-2 FTCA and 8-2 FTUCA which are known to be transient metabolites of 8-2 FTOH (Dinglasan et al. 2004; Fasano et al. 2006; Martin et al. 2005). Based on the articles by Martin et al. (Martin et al. 2002) and Ellis et al. (Ellis and Mabury 2003), initial trials in our laboratory utilized negative chemical ionization (NCI) GC/MS for the detection of fluorotelomer-based chemicals. While NCI allowed more sensitive detection limits for the 8-2 FTOH (7 ppb as reported by Martin et al. (Martin et al. 2002) and approximately 4 ppb in our efforts), chromatography could not resolve the perfluorinated acids and/or their methyl ester counterparts using chemical ionization. Using GC(NCI)/MS for the analysis of both

PFOA-me and PFNA-me resulted in detection limits greater than those established for the current EI method (data not shown).

Although this method results in IDLs and MDLs higher than literature reported values of non-volatile PFCs, it has the advantage of simultaneously quantifying both volatile and non-volatile fluorotelomer-based chemicals. In addition, monitoring perfluoroalkyl parent chain ions with GC/MS also has the ability to identify unknown metabolites by coupling other derivatization techniques. Disadvantages include higher IDLs and MDLs for non-volatile PFCs and extended sample preparation (approximately 45 min/ 8 samples). However, this method can also be used as a confirmatory method to LC-MS/MS, and offers a reliable alternative method of fluorotelomer-based chemical analysis by small-scale laboratories.

Method Development and Characterization (GC/MS Analysis): Initially, electron ionization (EI) spectra of the PFCs were generated over the range of 40 to 500 atomic mass units (amu), for 8-2 FTOH, the methyl esters of PFOA and PFNA, the methyl esters of the intermediate compounds (8-2 FTCA and 8-2 FTUCA), and the internal standards (PFHA-Me and 3-PFOP). Using the chromatographic conditions outlined in the methods section, fragment ions suitable for detection and quantification with GC/MS were selected. Each of the spectra contained the deprotonated molecular ion, but in low abundance, and therefore, quantification based on these ions would result in higher limits of detection. Upon investigating the fragmentation patterns, the majority of ions were less than 150 *m/z* as reported by Szostek and Pricket (Szostek and Prickett 2004). However, quantification was based on ions determined to be common among PFCs and included ions of *m/*z 131, 169, 231, 331 (Figure 3-1). Chemical structures associated with these ions are C_3F_5, C_3F_7, C_5F_9 and C_7F_{13}, respectively, and are expected to include unsaturated-secondary and saturated-primary perfluorinated carbonium ions.

These ions were formed by fragmentation of the perfluoroalkyl tail associated with the compounds of interest in 8-2 FTOH metabolism and degradation. Furthermore, it is also hypothesized that monitoring for these common ions allows identification of currently unknown structurally-related, volatile fluorotelomer-based chemicals.

The method was tested with FTOHs ranging from 8 to 12 carbons (6-2 FTOH to 10-2 FTOH) at 500 ng/mL and PFCA-me's ranging from 6 to 12 carbons at 250 ng/mL (data not shown), all with fragmentation patterns containing the perfluoroalkyl chain ions. The range of PFCA-me's tested, and commercially available FTOHs, all contained the desired mass fragments except the shorter chained perfluorohexanoic acid (PFHxA) and 4-2 FTOH. These two chemicals, due to smaller molecular weight, are absent of mass fragment 331 *m/z* and were quantified based on ions 131, 169, and 231 *m/z*. Because of PFHxA's increased volatility as a short-chain methyl ester, the initial hold time (1 min at 60 ºC) had to be increased to 3 min to prevent PFHxA from eluting with the solvent front. Instrumental response, based on area under the curve, increases for both homolog series of PFCA-me's and FTOHs, reaching maximum values when 10 carbons are present and plateaus for longer chain compounds. Peak shouldering occurred early in the method run (PFHxA-me and PFOA-me) and can be attributed to the 5 ºC/min ramp. This effect was experimentally corrected by incrementally increasing this rate; however, peak separation for compounds eluting later in the chromatography was compromised. Therefore integration was conducted including the peak shoulders and correlation coefficients (r^2) exceeded 0.99 (Table 3-1).

When examining the relative abundance of the ions monitored, 131 and 231 *m/z* increase with increasing chain length in PFCAs, while ion 331 *m/z* abundance decreases (Figure 3-2). An increasing trend was apparent with ion 169 *m/z* in the group of FTOHs examined while the

abundance of 231 and 331 *m/z* remained constant. The EI spectra of 6-2 FTOH and 8-2 FTOH have been previously investigated by Napoli and co-workers (Napoli et al. 1994). In agreement with their research, similarly dominant, structurally-specific ions were noted for the FTOHs. The developed method was compared with GC/MS conditions relying on the chemical-specific, parent ions (Figure 3-1). Based on area under the curve, the method monitoring for mass fragments 131, 169, 231, and 331 *m/z* allowed for greater sensitivity and lower detection limits than previously established analytical techniques. Thus, quantification of each analyte was calculated using the area under the curve for all ions monitored. Furthermore, quantification based on the ion with the largest abundance (*m/z* 131 for all PFCs) was investigated. When all ions were normalized to mass fragment 131 *m/z*, similar trends were noted and quantification and confirmation can be based on two qualifying ions (*m/z* 169 and 231) in ratio to the quantifying ion (*m/z* 131) coupled with standard retention times in each matrix (Figure 3-2). Specificity can be added by monitoring for ions 59 or 95 *m/z* that are characteristic for methyl esters and alcohols and to further confirm compound classes.

Detection Limits and Calibration: Based on United States Environmental Protection Agency (U.S. EPA) guidelines (40 CFR part 136, Appendix B.), IDLs were defined as the concentration (ng/mL) equivalent to three times the standard deviation of replicate instrumental measurements of the target analyte in MTBE. To establish the method quantification limit, a minimum of seven samples of each matrix (serum and liver), at both the low and high spike levels, were processed through the entire preparation and analytical method; including ion-pair extraction and derivatization with diazomethane. The standard deviation (σ) of the replicates was computed, and the MQL was determined by multiplying σ times a Student's t-value (STV) appropriate for a 99% confidence level (i.e., n = 7, STV = 3.143). Instrument detection limits for

all of the target analytes were determined to be in the low ng/mL range (Table 3-1). Average IDLs for FTOH target analytes in MTBE ranged from 5.6 to 9.0 ng/mL, the PFCA-me analytes in MTBE ranged from 4.5 to 7.6 ng/mL, and the FTCAs ranged from 7.1 to 24.5 ng/mL in MTBE (Table 3-1). Calibration data were then obtained on a linear range from 5 to 500 ng/mL depending on the analyte, using at least 5 standards for each calibration. All analytes had an r^2 value greater than 0.99. Method quantification limits for each analyte, calculated at both 25 and 200 ng/mL serum or ng/g liver, are reported in Table 3-2.

Within-day precision was tested by injecting 10 replicate samples (25 ng/mL or ng/g) processed through extraction and derivatization, to compare repetitive instrumental response and to further validate % extraction and derivatization. Between-day precision of GC/MS analysis was determined by repeatedly analyzing the samples from above over a 7 d period (n = 5 replicates/d for days 1, 5 and 7). The coefficient of variation for between-day precision was slightly greater than within-day most notably when comparing values for 8-2 FTOH, 8-2 FTCA and 8-2 FTUCA (Table 3-2). This is expected due to the stability of the intermediate acid metabolites and the volatility of 8-2 FTOH (Loewen et al. 2005).

Standard solutions were also made in methanol, MTBE and acetonitrile to determine which solvent worked best for the developed chromatographic conditions. As published by Szostek and Prickett (Szostek and Prickett 2004), solvent selection impacted the response, baseline stability, and signals to noise (S/N) ratios obtained. MTBE resulted in minimal background/solvent interference, sharp analyte peaks and reproducibility compared to the other solvents (data not shown). MTBE was also the preferred solvent due to its use in the ion-pair extraction method modified by Hansen et al. (Hansen et al. 2001) and as a diazomethane solvent in the derivatization step.

Method Performance (Extraction and Derivatization): Percent derivatization experiments were conducted using MTBE as the ethereal trap for diazomethane in solvent blank, and in serum and liver homogenates. To determine percent derivatization the following were processed with diazomethane as described above: PFHA, PFOA, PFNA, 8-2 FTCA and 8-2 FTUCA. PFHA, PFOA and PFNA methyl ester standards were used in calculating derivatization for the two perfluorocarboxylates. Standard solutions of the carboxylic acids and other PFCAs of interest were prepared (see Materials and Methods), derivatized and the area under the curve compared to that of their respective methyl ester (me) standards (Table 3-3). Percent derivatization increased as the carbon chain decreased for the series of PFCAs investigated. Because PFHA-me was the shortest chain PFCA tested and coupled with the improved performance of the modified derivatization technique employed, PFHA-me was used as an internal standard to more accurately reflect concentrations of PFOA and PFNA and the telomer acids in our samples.

As expected (due to minimal matrix interference), percent derivatization was greatest in MTBE blanks. The complexity of the tissue (liver more complex than serum), aids in explaining the decrease in % derivatization in the liver. Increasing the derivatization time for the liver extracts (≥45 minutes) increased derivatization performance.

Several methodologies for the production of diazomethane were evaluated (data not shown). The formation of diazomethane by reacting N-methyl-N-nitro-N-nitrosoguanidine (MNNG) with sodium hydroxide (NaOH) resulted in significantly lower percent derivatization than did the method utilizing Diazald® (Ngan and Toofan 1991). Derivatization for all compounds exceeded 90%, which is in agreement with those published by Ngan and Toofan (Ngan and Toofan 1991). Average percent derivatization for each compound is listed in Table

3-2. Previously mentioned temporal modifications decreased standard error across matrices to within 5% (data not shown). Furthermore, percent derivatization (Table 3-3) for 8-2 FTOH and 3-PFOP is considered the amount remaining after derivatization when compared to standard solutions.

The methyl esters of the intermediate metabolites are not commercially available; therefore percent derivatization was not evaluated for these compounds. Extraction and recovery were estimated for 8-2 FTCA and 8-2 FTUCA from standard curves constructed from derivatized (assumed 100%) standards. Formation of the FTUCAs from their corresponding FTCAs was shown to occur in methanol and water by Loewen et al. (Loewen et al. 2005); therefore, consideration must be given when quantifying these compounds to avoid possible confounding effects due to experimental conversion. It was assumed that heat generated by the reaction with diazomethane resulted in conversion of 8-2 FTCA to 8-2 FTUCA (approximately 8%); however, heat generated did not significantly reduce the yield of the other analytes.

Mammalian Matrices Spike-Recovery Experiments: Extraction efficiencies for each matrix are presented in Table 3-3 as are the data obtained from method validation in mouse serum and liver homogenates. Recovery in mammalian matrices exceeded 81% for all PFCs investigated. Hansen et al. (Hansen et al. 2001) first reported utilization of ion-pair extraction for PFOA determination in mammalian matrices, for both serum and liver. For PFOA, compiled extraction efficiencies for serum and liver averaged 101 and 87%, respectively (Hansen et al. 2001). In agreement with the results of Hansen et al. (Hansen et al. 2001), extraction efficiencies for perfluorocarboxylates in this study were greater in serum than liver, and percent extraction decreased with increasing perfluoroalkyl chain length.

Quantitative Analysis of 8-2 FTOH Metabolism: Following the procedure outlined above and based on the proposed metabolism pathway of the 8-2 FTOH, GC/MS analysis was conducted for 8-2 FTOH, 8-2 FTCA, 8-2 FTUCA, PFNA, and PFOA. Concentrations of 8-2 FTOH, 6 h after treatment in both serum and liver were 97±26 ng/mL and 134±42 ng/g, respectively. Several studies have illustrated that the metabolism of 8-2 FTOH is rapid and most of the parent compound is biotransformed at 6 h post-treatment *in vivo* and between 1-3 h *in vitro* (Kudo et al. 2005; Martin et al. 2005). Martin and coworkers determined that at 4 h post-treatment *in vitro*, 78% of the parent FTOH had been metabolized; however, metabolites only accounted for approximately 8.5% total molar mass (Martin et al. 2005). Concentrations of metabolites present exhibited the following trend; 8-2 FTUCA > 8-2 FTCA > PFOA > PFNA (Martin et al. 2005). In serum, the concentration of PFOA was 972±44 ng/mL and 277±29 ng/g in liver while PFNA concentrations were 65±15 ng/mL and 60±22 ng/g, respectively. All data was validated with LC/MS-MS using the method of Powley et al. (Powley et al. 2005) with less than 20% variation.

Method Significance: The present study describes a method for the simultaneous determination of several FTOHs and PFCA-me's by a GC/MS analysis in SIM mode. A selective and sensitive method for analysis of perfluorinated chemicals (PFCs) in both environmental and mammalian tissue matrices included monitoring for mass fragments 131, 169, 231, and 331 *m/z*, which are all common to the perfluorinated alkyl chains of this class of compounds. The IDL of the method ranges from 4.5 to 24.5 ng/mL, depending on the analyte. The average IDL for FTOHs and terminal PFCA-me's averaged 5.1 ng/mL (MTBE) while the MQL for all analytes was less than 50.0 ng/mL (ng/g). This study demonstrates that this method can be used to measure perfluorinated compounds in environmental samples and

mammalian matrices. Although average IDLs and MQLs are higher than currently reported methods, this method has the advantage of simultaneously detecting both volatile and non-volatile fluorotelomer-based chemicals in complex matrices.

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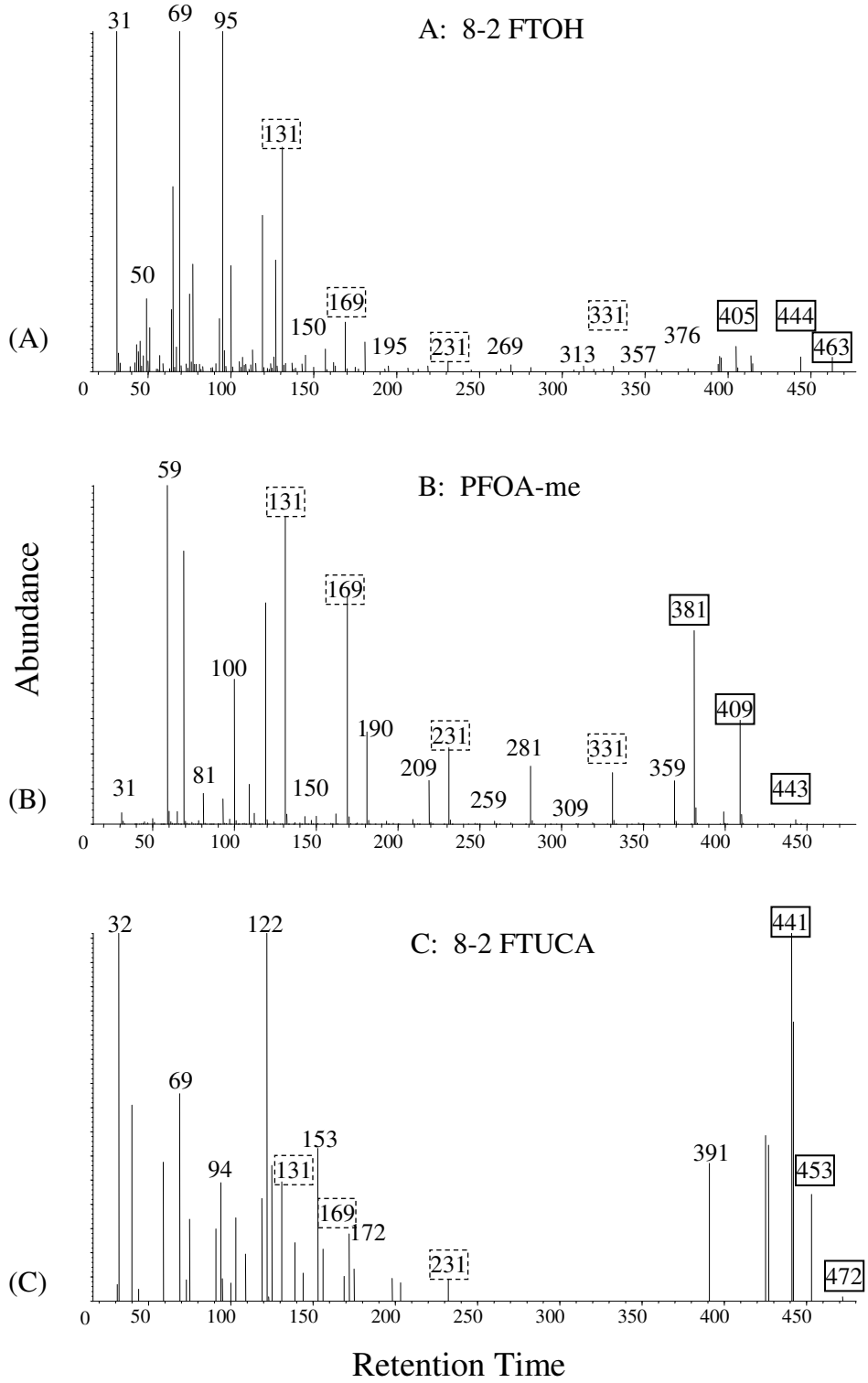

Figure 3-1: Representative mass spectra of 8-2 FTOH (A), and the derivatized metabolites PFOA-me (B), and 8-2 FTUCA-me (C). Chemical-specific ions, structurally related to each compound, are denoted by solid boxes and those common to the group of PFCs investigated in this study are illustrated by dashed squares.

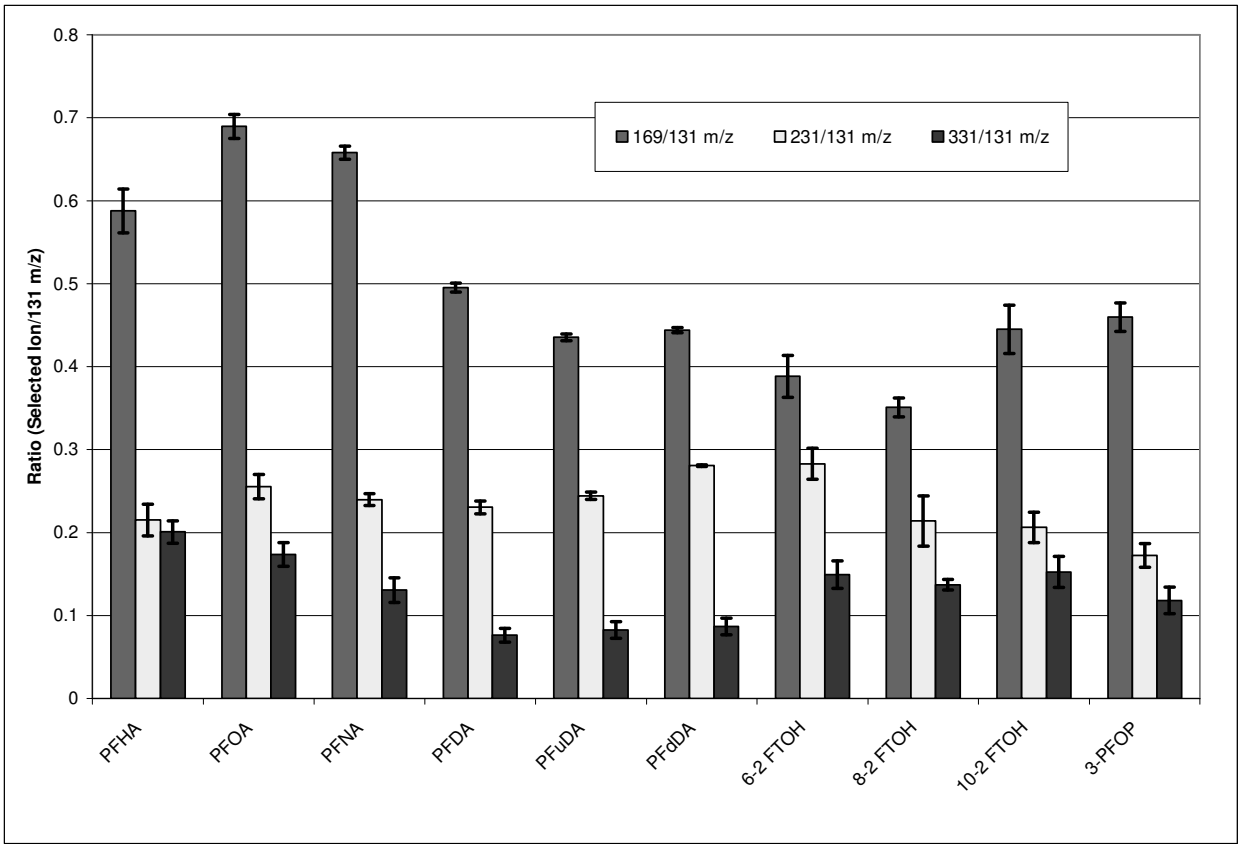

Figure 3-2: Ratio of average abundance of ions 169, 231, and 331 *m/z* normalized to 131 *m/z* for PFHA-me (C7 acid methyl ester) through PFdDA-me (C12 acid methyl ester) and the 6-2, 8-2, and 10-2 FTOHs in MTBE. 3-PFOP and PFHA-me are used as internal standards for the FTOHs and FTCA-me's, respectively.

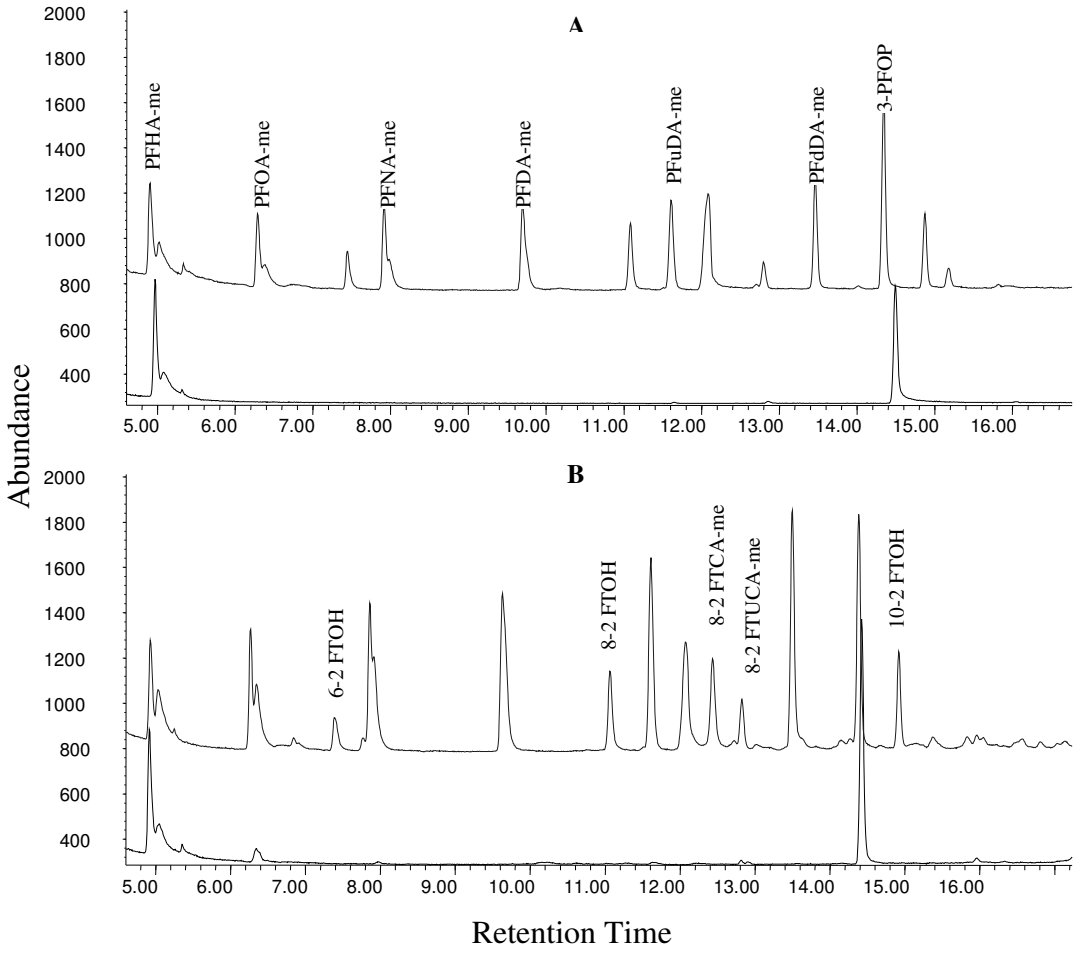

Figure 3-3: Representative chromatograms of serum (A) and liver (B) spike-recovery experiments, including matrix blanks (bottom plot), using the developed method for monitoring fragments 131, 169, 231, and 331 *m/z*. Internal standards and PFCA-me's are labeled in (A) and FTOHs and intermediate 8-2 FTCA-me's identified in (B). Chromatographic plots have been offset by a factor of +500 units to aid in visualization of peak height and area under the curve differences.

Table 3-1: Fragmentation patterns, retention times, and method validation parameters for PFCs implicated in 8-2 FTOH metabolism.

Compound	Molecular Weight (amu)	RT (minutes) [a]	Calibration Range (ng/mL); r^{2} [b]	Estimated IDL [c] (ng/mL)
8-2 FTOH	464.12	11.11	5-500; 0.9908	5.6
8-2 FTCA-me	478.11	12.08	5-200; 0.9916	24.5
8-2 FTUCA-me	458.10	12.83	5-200; 0.9959	7.1
PFNA-me	478.11	7.90	5-500; 0.9977	4.5
PFOA-me	428.10	6.28	5-500; 0.9924	5.5
PFHA-me [d]	378.09	4.90	10-250; 0.9912	7.6
3-PFOP [d]	478.14	14.91	10-250; 0.9935	11.2

[a] Retention time of chemical under chromatographic conditions outlined above.
[b] r^2 - Correlation coefficient for regression analysis over calibration range
[c] Instrumental Detection Limit - concentration equivalent to 3 times the standard deviation of replicates ($n \geq 7$) in reagent blank (MTBE).
[d] PFHA-me and 3-PFOP were used as internal standards for the PFCA-me's and FTOHs, respectively.

Table 3-2: Method performance parameters for volatile and non-volatile PFCs extracted from serum or liver samples.

Compound	Estimated MQL[a]		Within-Day Precision (COV)[b]	Between-Day Precision (COV)[c]
	Low[d]	High[e]	Low	Low
8-2 FTOH	15.6	22.8	2.7	13.8
8-2 FTCA	ND[f]	49.5	5.6	8.6
8-2 FTUCA	ND[f]	25.4	9.6	11.3
PFNA	13.9	17.3	2.9	8.0
PFOA	9.3	11.9	3.3	8.0
PFHA[g]	18.1	21.3	5.0	5.8
3-PFOP[g]	17.3	16.8	7.2	9.0

[a] Method Quantification Limit – concentration equivalent to 3 times the standard deviation of replicates processed through the entire method ($n \geq 7$) appropriate for a 99% confidence interval. MQL presented is the average from both serum and liver samples.
[b] COV - Coefficient of variation for within-day accuracy ($n \geq 7$) calculated as the average of serum and liver values at the low spike level.
[c] COV - Coefficient of variation for between-day accuracy over a period of 7 d (n = 5 replicates/d for days 1, 5 and 7) calculated as the average of serum and liver values at the low spike level.
[d] Low = Target spike level of 25 ng/mL serum or ng/g liver.
[e] High = Target spike level of 200 ng/mL serum or ng/g liver.
[f] ND = Not determined because the MQL's were above 25 ng/mL (ng/g) spike-level and were therefore calculated based on 200 ng/mL (ng/g) concentration.
[g] PFHA-me and 3-PFOP were used as internal standards for the PFCA-me's and FTOHs, respectively.

Table 3-3: Validation parameters for volatile and non-volatile PFCs extracted from both serum and liver samples.

Compound	% Derivatization[a]	% Recovery (Serum)[b]	% Recovery (Liver)[b]
8-2 FTOH	91 ± 3.5[c]	88 ± 6.7	84 ± 6.3
8-2 FTCA	ND[d]	91 ± 4.9	89 ± 2.6
8-2 FTUCA	ND[d]	93 ± 1.9	81 ± 5.9
PFNA	92 ± 3.6	98 ± 2.4	89 ± 8.3
PFOA	98 ± 4.1	101 ± 2.1	90 ± 3.7
PFHA[e]	114 ± 9.2	84 ± 5.6	81 ± 4.8
3-PFOP[e]	99 ± 6.4[c]	92 ± 3.7	90 ± 6.1

[a] Average % derivatization for n = 10 replicates ± standard deviation.
[b] Average % recovery for n = 10 spiked replicates for each matrix sample ± standard deviation at the 25 ng/mL spike level.
[c] % derivatization for 8-2 FTOH and 3-PFOP is considered amount surviving derivatization conditions.
[d] ND = Not determined because standards are not currently commercially available.
[e] PFHA-me and 3-PFOP were used as internal standards for the PFCA-me's and FTOHs, respectively.

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CHAPTER 4

DISTRIBUTION OF 8-2 FLUOROTELOMER ALCOHOL (FTOH) AND ITS OXIDATION METABOLITES IN MALE CD-1 MICE [2]

[2] Henderson, W. Matthew, Eric J. Weber, and Mary Alice Smith. 2006. To be submitted to Toxicological Sciences.

Abstract

8-2 Fluorotelomer alcohol (FTOH), a primary compound used in polymer synthesis, is found ubiquitously in the environment and can undergo biotic and abiotic transformation to perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA). The global occurrence of both PFOA and PFNA and their potential toxicity has spurred regulatory interest in potential sources for these compounds. An appropriate animal model is needed to elucidate 8-2 FTOH metabolism and distribution of its metabolites in mammals. The objective of this study was to investigate the metabolism of 8-2 FTOH in male CD-1 mice. Mice received a gavage dose (30 mg 8-2 FTOH/kg BW) in a propylene glycol/water (1:1) vehicle, animals were serially sacrificed and tissues excised for analysis via GC/MS. 8-2 FTOH was rapidly metabolized and no longer detected by 48 hrs post-treatment and the estimated half-life of 8-2 FTOH in serum of male mice was 6.2 hrs. The intermediate metabolites, 8-2 fluorotelomer carboxylic acid (FTCA) and 8-2 fluorotelomer unsaturated carboxylic acid (FTUCA) were detected in both serum and liver at the earliest time-point investigated (0.5 hours). 8-2 FTUCA was the most abundant intermediate metabolite and concentrations of 8-2 FTCA were approximately 1.5-fold less than 8-2 FTUCA concentrations in both serum and liver. Maximal PFOA concentrations, a terminal metabolite, in serum (992±38 ng/mL) and liver (902±27 ng/g) occurred at 12 hrs post-treatment and then decreased for up to 120 hrs. The highest concentration of PFNA coincided with peak PFOA concentrations and maximal concentrations of the terminal metabolites were detected in serum with estimated half-lives of 14.9 and 11.8 days, respectively. In conclusion, these results suggest 8-2 FTOH is metabolized by both alpha- and beta-oxidation pathways in mice, which unlike rats, do not rapidly excrete PFOA and PFNA. The abundance of PFOA at 10-times that of PFNA suggests that β-oxidation is the predominant pathway.

Introduction

Fluorotelomer-based chemicals such as the fluorotelomer alcohols have been identified as indirect sources of numerous stable perfluorinated chemicals identified in mammalian and environmental matrices (Hagen et al., 1981; Kudo et al., 2005; Martin et al., 2005; Fasano et al., 2006) (Table 4-1). Thus, it has been hypothesized that volatile perfluoroalkyl telomers, namely 8-2 telomer alcohol (FTOH), are released during preparation of telomer-based products and subsequently metabolized into PFOA and PFNA (Ellis et al., 2004; Stock et al., 2004). The FTOHs are used in both industrial and commercial applications to impart water, heat and acid resistance as well as surface-protective features (Mylchreest, Munley and Kennedy, 2005; Prevedouros et al., 2006).

PFCs, namely the perfluorocarboxylic acids (PFCAs), perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA) as well as their sulfonic acid analogue, perfluorooctane sulfonate (PFOS) represent an emerging class of persistent organic pollutants due to their resistance to mammalian metabolism and environmental biodegradation (Prevedouros et al., 2006; Renner, 2006). While these stable acids are detected worldwide, current estimated direct releases are not able to account for concentrations detected in both mammalian and environmental matrices and efforts are aimed at determining possible indirect sources such as metabolism and degradation of precursor compounds.

Of particular concern is the environmental and mammalian bioavailability of PFOA and PFNA. Both PFCAs have been shown to be globally distributed (Giesy and Kannan, 2001; Martin et al., 2002; Kannan et al., 2005), persistent (Martin et al., 2004; Andersen et al., 2006; Smithwick et al., 2006), bioaccumulative (Tomy et al., 2004), and potentially toxic (Butenhoff et al., 2004; Lau, Butenhoff and Rogers, 2004; Kudo et al., 2005). Because it is unlikely to be

transported once deposited, it is suggested that PFOA should remain at or near the source. However, PFOA has been detected in polar-bear blood samples from the arctic region (Smithwick et al., 2005a; Smithwick et al., 2005b) and in 90 percent of voluntary human samples tested (Olsen et al., 2003a; Emmett et al., 2006b).

The toxicology of PFCAs has been reported with a wide variety of effects, such as inhibition of gap junction intercellular communication (Hu et al., 2002) to hepatic tumor promotion (Biegel et al., 2001) and peroxisome proliferation (Berthiaume and Wallace, 2002; Kudo et al., 2005). PFOA is reported to have carcinogenic effects in rats (Biegel et al., 2001) due to its actions as a peroxisome proliferator as well as general developmental toxicity in both rats and mice (Lau et al., 2004; Lau et al., 2006). This evidence has prompted regulatory action to determine the potential human-health impact of perfluorinated chemicals (USEPA, 2001).

Although PFOA is used in the manufacture and synthesis of fluoropolymers, it has been postulated that direct industrial releases coupled with the environmental metabolism and biodegradation of precursor compounds results in the concentration of PFCAs detected in matrices worldwide (Stock et al., 2004; Martin, Mabury and O'Brien, 2005). Plausible sources for the environmental availability of FTOHs and other precursor compounds include release from industrial and consumer applications (Dinglasan-Panlilio and Mabury, 2006; Prevedouros et al., 2006), residuals in consumer products due to incomplete covalent linkages during polymer production (Dinglasan-Panlilio and Mabury, 2006), and thermally-mediated polymer degradation (Ellis et al., 2004; Yamada et al., 2005; Dinglasan-Panlilio and Mabury, 2006).

Mammalian metabolism as seen by Hagen et al. (1981) and in Martin et al. (2005) suggests that exposure to precursor compounds may help account for the PFCAs determined in human blood samples (Olsen et al., 2003b; Olsen et al., 2004; Olsen et al., 2005; Emmett et al.,

2006a). Furthermore, isomeric profiles seen in wildlife (ie. polar bears) suggest that the concentration of straight-chain perfluorinated carboxylic acids detected originate from precursor, straight-chained compounds such as the FTOHs (Martin et al., 2004; Smithwick et al., 2005b).

The purpose of this study was to investigate single, low-dose oral exposures (10, 20 or 30 mg 8-2 FTOH/kg BW) and the subsequent mammalian metabolism and distribution of 8-2 FTOH in an appropriate animal model. The metabolic parameters of 8-2 FTOH in rats have been studied (Figure 4-1); however, limited information is available on metabolism of the 8-2 telomer alcohol in mice. The objective of this study was to determine the metabolism and distribution of 8-2 FTOH and its oxidation metabolites in male CD-1 mice. We report quantitative information on intermediate (8-2 FTUCA and 8-2 FTCA) and terminal (PFOA and PFNA) metabolite formation, retention and total systemic exposure to these metabolites following 8-2 FTOH exposure. Understanding the contribution of mammalian metabolism of 8-2 FTOH will aid in explaining the role of precursor compound exposure to the concentrations of PFC metabolites detected worldwide.

Materials and Methods

Chemicals: All chemicals and reagents used in this study were obtained as highest purity available (>98%), as determined by the supplier. Perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA) and 8-2 flurotelomer alcohol (8-2 FTOH; >98% purity) were purchased from Oakwood Research Chemicals (West Columbia, SC). Both the 8-2 fluorotelomer acid (8-2 FTCA) and the 8-2 fluorotelomer unsaturated acid (8-2 FTUCA) were purchased from Wellington Laboratories (Toronto, Canada). The methyl esters of PFOA and PFNA (PFOA-me and PFNA-me) were also obtained from Oakwood Research Chemicals and

used as comparative standards to the methyl esters prepared by derivatization with diazomethane for detection and quantification of these chemicals by gas chromatography-mass spectroscopy (GS/MS). Perfluoroheptanoic acid (PFHA) and 8-2 telomer propanol, obtained from Oakwood, were used as internal standards for the acid methyl esters and the alcohols, respectively. The isotopic standard, mass-labeled PFOA (mPFOA) was purchased from Wellington Laboratories (Toronto, Canada) and used as an internal standard for validation and re-quantification by LC-MS/MS.

Animal Studies: Male CD-1 mice were purchased from Charles River Laboratory (Raleigh, NC). Before initiating the study, animals were allowed to acclimate for 7 days in micro-isolator shoebox cages (up to 5 animals per cage). The cages were housed in constant temperature and constant humidity controlled rooms with a 12 hour light/dark cycle. Animals were fed Lab Diet Certified Rodent Chow with both food and water provided *ad libitum*. All animal studies were conducted in accordance with animal welfare regulations and were approved by the University of Georgia's Institutional Animal Care and Use Committee (IACUC).

Mice were gavaged with 8-2 fluorotelomer alcohol in a 1:1 mixture of deionized water: propylene glycol at doses of 10, 20 or 30 mg 8-2 FTOH/kg body weight (BW). Dose concentrations were confirmed by GC/MS analysis. Dose volumes were calculated not to exceed 0.5 mL/mouse. Animals were serially sacrificed at 0.5, 1, 3, 6, 12, 24, 48 and 120 hours post-treatment and organs excised for PFC analysis via GC/MS and LC-MS/MS.

Sera and Tissue Collection: Following CO_2 asphyxiation, whole-blood was collected via cardiac puncture and placed in 1.2 mL centrifuge tubes on ice prior to centrifugation 600 xg for 15 minutes at 20ºC. Serum was removed and transferred to a new tube and stored at -80ºC prior to fluorochemical quantification. Liver, kidney, gonad, fat, spleen and brain tissues were

excised, and weighed on a Mettler PM4000 balance and immediately flash frozen with a combination of liquid nitrogen and dry ice and stored at -80ºC until analyzed. To prevent the possible evaporative loss of 8-2 FTOH and volatile intermediates, all tissue samples were immediately capped and remained frozen until analyzed. Similarly, whole-blood was capped and contained on wet-ice until freezing after centrifugation.

PFC Extraction: PFCs from serum and tissue were extracted using a previously published method by ion-pair extraction with slight modifications (Hansen et al., 2001). The method enabled concurrent liquid-liquid extraction of the FTOHs with ion-pair extraction of the FTCAs and PFCAs. Briefly, 250 μL of serum was added to 1 mL of 0.5 M tetra butyl ammonium hydrogen sulfate (TBAHS) solution (adjusted to pH 10), 2 mL of 0.25 M sodium carbonate buffer and 5 μL of internal standard in a 15 mL polypropylene tube. After thorough mixing, 2.0 mL methyl tertiary-butyl ether (MTBE) was added to the solution, which was then vortexed for 15 minutes. Finally, the mixture was centrifuged for 10 minutes and 1.75 mL of the organic layer was removed for PFC analysis.

Similarly, tissue extractions were performed using the modified ion-pair extraction and liquid-liquid extraction techniques. For the extraction of liver samples, a liver homogenate of 1.0 gram of liver to 1.0 mL of Milli-Q water was prepared using an electric homogenizer. One-half mL of the homogenate was transferred to a new polypropylene tube containing the same reagents as serum extraction (see above). Extractions were performed for 0.5 grams of kidney, gonad or fat. Samples for spleen and brain were combined within treatment groups to achieve similar masses ($n \geq 3$; 0.5 grams). Furthermore, each tissue was homogenized in an equal volume of Milli-Q water prior to ion-pair/liquid-liquid extraction and derivatization.

For derivatization, the organic layer (0.75 mL) was then added to 0.25 mL of ethereal-trapped diazomethane generated with a Wheaton micro-generator (Aldrich Chemical Company, Milwaukee, WI) by reacting Diazald® with potassium hydroxide (KOH) as described by Ngan and Toofan.

PFC Analysis (GC/MS): PFC analysis with GC/MS was conducted using a previously published method (Henderson et al., 2006). Briefly, samples were analyzed using a Hewlett Packard (HP) 6890 Series Gas Chromatograph equipped with a HP 5973 Mass Spectrometer and a HP 6890 Series Injector (Palo Alto, CA). The GC was equipped with a Restek (Bellefonte, PA) RTX200MS (30m x 0.25 mm ID, 0.5 μm df) trifluoropropylmethyl polysiloxane phase column. Samples were introduced (2 μL) into the injector operating in splitless injection mode (injector temperature 225ºC) and separated chromatographically using a temperature program of an initial oven temperature of 60ºC ramped at 3ºC/minute to 150ºC then 50ºC/minute to 250ºC and held for 5 minutes. The mass spectrometer was operated in selective ion monitoring (SIM) mode with masses 131, 169, 231, and 331 *m/z* (Henderson et al., 2006). Furthermore, all data points were confirmed and quantified with LC/MS-MS using the method of Powley and co-workers (2005), incorporating mass-labeled PFOA as the internal standard (Powley et al., 2005).

Statistical Analysis: All statistical analysis was conducted with Statistical Analysis Software (SAS) (Cary, NC) and a Students t-test p-value of 0.05 was used as the level of desired significance. Data presentation and analysis was performed with Sigma Plot Version 9.0 (Systat Software, Inc., Point Richmond, CA) as were parameter calculations for area under the concentration curve from 0 to n-1 (AUC_{obs}) and the half-lives ($t_{1/2}$) following maximal observed concentrations (C_{max}).

Results

8-2 FTOH Metabolism Following Oral Exposure: Based on the proposed metabolism scheme of 8-2 FTOH (Figure 4-1), sera and tissues were analyzed for the parent compound (8-2 FTOH), selected intermediate metabolites (8-2 FTUCA and 8-2 FTCA), and the terminal metabolites, PFOA and PFNA. In male mice, 8-2 FTOH rapidly decreased in serum. At 24 hours post-treatment, a minimal amount of 8-2 FTOH was detected and by 48 hours, 8-2 FTOH could not be detected in serum irrespective of dose group (Figure 4-2A).

Oxidation of 8-2 FTOH is illustrated by the appearance of the metabolites 8-2 FTUCA and 8-2 FTCA following oral exposure to 8-2 FTOH (Figure 4-2B and 4-2C). While 8-2 FTOH concentrations were decreasing, 8-2 FTUCA and 8-2 FTCA were approaching maximal concentrations at 1 hour post-treatment and subsequently, decreased up to 24 hours. 8-2 FTUCA is the most abundant metabolite likely due to its formation via –HF reduction from both 8-2 FTCA as well as 8-2 FTUAL (not identified in the present study). In comparing 8-2 FTUCA to 8-2 FTCA, concentrations were approximately 2.5-fold less than 8-2 FTUCA concentrations. 8-2 FTUCA can also undergo reduction to form 7-3 FTCA although concentrations of 7-3 FTCA, were similar in magnitude to 8-2 FTCA suggesting that PFOA formed by this route of oxidation is minimal (data not shown).

At 5 days post-treatment, approximately 4% of 8-2 FTOH is transformed into the terminal metabolites PFOA and PFNA (Figure 4-2D and 4-2E). PFOA reaches maximal concentrations by 12 hours post-treatment coinciding with decreased parent (8-2 FTOH) and decreasing intermediate (8-2 FTUCA and 8-2 FTCA) metabolite concentrations. By 6 hours post-treatment, concentrations of PFOA from the three treatment groups increase 1.5-fold with increasing amount of 8-2 FTOH administered (Figure 4-2B). In contrast, PFNA reaches

maximal concentrations 24 hours post-treatment and is below method quantification limits at time-points less than 3 hours. PFOA is the most abundant terminal metabolite suggesting that β-oxidation occurs in the CD-1 mouse. Based on concentrations of 8-2 FTUCA, in the absence of conjugate data, the majority of PFOA appears to be formed by oxidation of 8-2 FTUCA (Figure 4-2B and 4-2D).

Distribution of 8-2 FTOH: Maximal 8-2 FTOH serum concentrations (C_{max}) are achieved before 0.5 hours ($t_{max(obs)}$) following treatment (Table 4-2). Log concentrations of 8-2 FTOH exponentially decrease with r^2 values of least squares regression ≥ 0.96 (Figure 4-2A) resulting in a $t_{1/2}$ of 6.2 days. The highest concentrations detected in serum at 0.5 hours post-treatment following 30 mg 8-2 FTOH/kg BW was 1633±48 ng/mL (Table 4-3). However, statistical differences in the amount of 8-2 FTOH present in serum were not achieved until 1 hour post-treatment when comparing 10, 20 and 30 mg/kg BW (Figure 4-2A).

At 0.5 hours post-treatment, 8-2 FTOH was most abundant in liver with concentrations approximately 1.5-fold greater than those determined in serum and this trend continued until 24 hours post-treatment (Figure 4-2A and 4-3). The highest concentrations detected in liver at 0.5 hours post-treatment following 30 mg 8-2 FTOH/kg BW was 2083±36 ng/g. Similar to serum concentrations, there was a dose dependency in the amount of 8-2 FTOH detected in liver (data not shown). Liver concentrations of 8-2 FTOH also temporally decrease exponentially with r^2 values of log-transformed, least squares regression ≥ 0.90 (Figure 4-3).

8-2 FTOH was not detected in the other rapidly perfused tissues analyzed (kidneys, gonads, spleen) until 1-3 hours post-treatment and concentrations were generally 10 fold less than those determined in serum and liver (Figure 4-4). Maximal concentrations determined in these tissues following 10 mg 8-2 FTOH/kg BW (kidney (121±18 ng/g), spleen (95±12 ng/g),

and gonads (97±22 ng/g)) were proportional to the amount of blood flow to these organs *in vivo*. Similar to serum and liver, concentrations determined in these tissues increased approximately 1.5-fold with increasing dose from 10 to 20 mg 8-2 FTOH/kg BW and from 20 to 30 mg 8-2 FTOH /kg BW (see supplemental data).

Interestingly, 8-2 FTOH was distributed to the brain and fat at the earliest time point analyzed (0.5 hours post-treatment). The concentrations in brain remained relatively constant at each dose level averaging 36±16, 54±13 and 68±19 ng/g in the 10, 20 and 30 mg 8-2 FTOH dose groups, respectively (see supplemental data). In contrast, the highest concentrations in fat were determined before 6 hours post-treatment and accounted for approximately 0.5% (0.2-1.0%) total dose, as no other metabolites were detected in fat (Table 4-3).

Distribution of Intermediate Metabolites: 8-2 FTUCA and 8-2 FTCA were approaching maximal concentrations at 1 hour ($t_{max(obs)}$) post-treatment and decreased until 24 hours post-treatment (Figures 4-2B and 4-2C, serum; Figure 4-3, liver). 8-2 FTUCA was found in the highest concentrations in tissue samples following treatment with 8-2 FTOH, and was generally 2-3 times more abundant than 8-2 FTCA. No intermediate metabolites (Figure 4-2B) were detected in serum following 24 hours post-treatment except in the 30 mg/kg BW dose group in which 8-2 FTUCA became near method quantification limits at or before 48 hours post-treatment (Figure 4-2C). Serum concentrations observed at $t_{max(obs)}$ summed for 8-2 FTUCA and 8-2 FTCA were comparable to serum concentrations of 8-2 FTOH supporting that 8-2 FTOH is rapidly metabolized in the mouse.

In liver, 8-2 FTUCA was the most abundant metabolite detected at 0.5 hours post-treatment and reached maximal concentrations at 1 hour post-treatment (Figure 4-3). Maximal concentrations of 8-2 FTCA occurred later (3-6 hours) than those determined for 8-2 FTUCA (1-

3 hours) and 8-2 FTCA concentrations are on average 3-4 fold less. Liver concentrations of both intermediate metabolites increase with increasing dose of 8-2 FTOH (Figure 4-3).

No appreciable amounts of either intermediate metabolite (8-2 FTUCA or 8-2 FTCA) were detected in kidney, brain, spleen, gonad or fat tissue at time-points analyzed (see representative kidney data, Figure 4-4).

Distribution of Terminal Metabolites: In serum, PFOA concentrations increase in a dose-dependent fashion with a C_{max} of 992±36 following a single oral dose of 30 mg 8-2 FTOH/kg BW (Figures 4-2D and 4-2E, Table 4-2). PFOA reaches maximal concentrations in serum at 12 hours post-treatment coinciding with decreased parent and decreasing intermediate metabolite concentrations (Figure 4-2D). Following 6 hours post-treatment, serum concentrations from the three dose groups increase 1.5-fold with increasing 8-2 FTOH exposure. PFNA reaches maximal concentrations 24 hours post-treatment and is not detected at time-points before 3 hours post-treatment (Figure 4-2E).

PFOA concentrations in liver are less than serum concentrations (Figure 4-3). Interestingly, PFOA concentrations in the liver initially increase (0.5-3 hours post-treatment), and decrease at 6 hours post-treatment before reaching maximal concentrations at 12 hours (Figure 4-3). Independent of dose, PFOA begins accumulating in the liver and reaches maximal concentrations at 12 hours. No statistical differences are present between the initial concentrations of PFOA (0.5-3 hours post-treatment) in liver following 10, 20 and 30 mg 8-2 FTOH treatments; however, statistical differences were noted 12 hours post-treatment (Figure 4-2D). Although less than peak serum concentrations, PFOA concentrations in the liver similarly decrease while PFNA concentrations in the liver exceed those of serum concentrations (serum, Figures 4-2D and 4-2E; liver, Figure 4-3).

Maximal terminal metabolite concentrations in the kidney occur concurrently with serum and liver concentrations, and concentrations account for approximately 0.1% of total dose administered. It should be noted that brain and fat were not analyzed for PFOA or PFNA; however, no appreciable amounts of these metabolites were detected in gonads or spleen extracts (see supplemental data).

Plasma Kinetics of 8-2 FTOH and Metabolites: Following 8-2 FTOH exposure, area under the curve (AUC) calculations were used to estimate total systemic exposure to parent compound and metabolites. Total area under the curve (8-2 FTOH) for observed (AUC_{obs}; 0.5-120 hours) and estimated to infinity (AUC_{∞}) were similar as were the estimated elimination rate constants (k_{el}) for serum (Table 4-2). The terminal half-lives ($t_{1/2}$) for 8-2 FTOH in serum were comparable across dose groups (Table 4-2). When plotting AUC_{∞} versus concentration, based on least squares regression ($r^2 \geq 0.98$), there is a dose dependency in total systemic exposure to 8-2 FTOH.

The estimated half-life ($t_{1/2}$) in serum for 8-2 FTUCA is 12-16 days and $t_{1/2}$ for 8-2 FTCA is 14-15 days (Table 4-2). Calculated AUC_{obs} and estimated AUC_{∞} values for both intermediate metabolites were of similar magnitude between dose groups; however, values determined for 8-2 FTCA were generally lower than 8-2 FTUCA (Table 4-2). Total AUC_{∞} for 8-2 FTUCA following 10, 20 and 30 mg 8-2 FTOH/kg BW increased from 6.67×10^3 to 1.43×10^4 ng·hr/mL and for 8-2 FTCA increased from 3.99×10^3 to 8.56×10^3 ng·hr/mL (Table 4-2). Therefore, increased exposure to 8-2 FTOH results in increased exposure to intermediate metabolites. Independent of dose, liver metabolism of 8-2 FTUCA is also exponential and linear regression ($r^2 \geq 0.95$) of log transformed data result in an estimated k_{el} of 1.0-1.5 ng·hr/mL (Table 4-2).

Of the compounds analyzed, PFOA and PFNA have the longest $t_{1/2}$'s ranging from 3-5 days when estimated post-C_{max}. Extrapolating PFOA and PFNA concentrations based on amount formed over time results in calculated $t_{1/2}$s of 15.1 and 14.9 days, respectively. The AUC_{obs} for PFNA are similar to its precursor 8-2 FTCA, suggesting that the dominant fate of 8-2 FTCA is metabolism into PFNA. However, due to its higher $t_{1/2}$, AUC_{∞} for PFNA is greater suggesting an alternate mechanism of metabolism may also be present (Table 4-2). Serum kinetic parameters for PFOA are also enumerated in Table 4-2.

Mass Balance: Following treatment with 8-2 FTOH and its subsequent metabolism, approximately 3-4% of total dose is recovered as PFOA and PFNA at 120 hours post-treatment (Table 4-3). The highest concentrations of 8-2 FTOH were determined at 0.5 hours post-treatment and accounted for <10% total dose. In the 10 mg 8-2 FTOH/kg BW dose group, >26% of the administered dose was absorbed compared to 12% in the 30 mg/kg BW dose group (see supplemental data).

Following 24 hours post-treatment, no parent compound was detected in any of the rapidly perfused tissues and approximately 0.3% resided in fat. At latter time-points (post-24 hours), PFOA is the most abundant metabolite and the percent dose recovered is higher in liver than serum. Irrespective of dose and metabolite concentration present, percent dose was greatest in the liver. However, at 6 hours post-treatment, the percent of dose recovered as metabolites in serum exceed calculated values in serum at latter time-points suggesting that intermediate metabolites can also be conjugated and/or excreted. Interestingly, overall percent dose accounted for by monitoring 8-2 FTOH, 8-2 FTCA, 8- FTUCA, PFOA and PFNA was highest at the lowest dose tested at all time points and as expected, decreased with increasing time (see supplemental data).

Discussion

Currently, little information is available on the metabolism and distribution of 8-2 FTOH in mammals. Due to the global occurrence and persistence of PFOA and PFNA, it is likely that exposure to precursor compounds account for the increasing concentrations being detected in both environmental and mammalian matrices. The most plausible explanation for global dissemination is that terminal acids are formed as metabolism products (Kudo et al., 2005; Martin et al., 2005) or as degradation products of several structurally-related homologues in the atmosphere coupled with processes responsible for β-oxidation under aerobic environmental conditions (Dinglasan et al., 2004; Wang et al., 2005a; Wang et al., 2005b). Coupling knowledge of both direct and indirect sources of exposure will ultimately complete the continuum of source to outcome for the human health risk assessment of perfluorinated chemicals.

Similarly, Hagen et al. (1981) first identified probable mammalian metabolism of 8-2 FTOH. 8-2 FTCA and PFOA were identified as metabolites in rats and it was hypothesized that 8-2 FTUCA was also an intermediate metabolite. This work was expanded and confirmed by both Martin et al. (2005) and Kudo et al. (2005) in which metabolites were tracked *in vitro* and *in vivo*, respectively. Because of this, we treated with 8-2 FTOH and tracked the parent compound, the stable intermediates (8-2 FTUCA and 8-2 FTCA), and the terminal metabolism products (PFOA and PFNA). PFOA and PFNA have been shown to be resistant to both metabolism and degradation processes and thus are the end products of 8-2 FTOH metabolism in mammals.

The results of this study in male CD-1 mice show that following a single oral dose, 8-2 FTOH is rapidly absorbed and cleared. This is similar to what has been reported in rats (Fasano

et al., 2006) suggesting that in both rats and mice 8-2 FTOH metabolism is rapid, occurring within 24 hours post-treatment. As in rats (Fasano et al., 2006), the most abundant metabolite detected in male CD-1 mice is 8-2 FTUCA and the half-lives of 8-2 FTUCA (4-18 hours) and PFOA (7-8 days) in male rats (Fasano et al., 2006) and male CD-1 mice is also similar.

Previous work in our laboratory (Henderson and Smith, 2006) reported the metabolism and distribution of 8-2 FTOH in pregnant and non-pregnant CD-1 mice. 8-2 FTOH oxidation resulted in the distribution of PFOA and PFNA similar to the current study in male mice and estimated half-lives for parent and terminal metabolites were not statically different between males and females. Therefore our data further support that mice lack the sex-mediated excretion processes observed in rats.

Interestingly, it is hypothesized that female rats rapidly excrete PFOA and other PFCs due to elevated isoforms of the group of organic-acid transporters (OAT). Rats possess an organic acid transport mediated excretion process that rapidly and efficiently decreases the body burden of PFOA (Hundley et al., 2006; Kudo et al., 2002), most notably in female rats and post-pubescent pups (Hinderliter et al., 2006). Humans are believed to be absent this anion excretion mechanism as well as gender-mediated excretion mechanisms of PFCs (Butenhoff et al., 2004b) and therefore rats are not an appropriate animal model for elucidating the human health risk of PFOA exposure. While gender differences are seen in rats, this sex-mediated route of elimination is thought to be absent in most other species (Houde et al., 2005; Kannan et al., 2002b; Smithwick et al., 2005).

The timing of metabolism and elevated concentrations of both intermediate metabolites (8-2 FTCA and 8-2 FTUCA) at 0.5 hours post-treatment suggests that metabolism in the gut may be occurring. Because PFOA and PFNA are known to be rapidly and completely absorbed

following oral exposure (Hundley et al., 2006), it is possible that the intermediate metabolites would also be rapidly absorbed. Irrespective of the metabolic processes involved in oxidation, the gut possesses both alcohol and aldehyde dehydrogenases and numerous cytochrome P450s as well as microbial activity. Gut metabolism is also supported by the initial surge of PFOA concentrations in the liver. However, delayed maximal PFNA concentrations in the liver suggest that unlike PFOA, 8-2 FTOH metabolism into PFNA is dependent on liver metabolism.

When examining the whole body-burden of precursor compound exposure in the rat, the majority (>70%) of 8-2 FTOH was excreted unchanged in feces exclusive of sex and dose level, 4% in urine of which approximately 1% was determined to be PFOA (Fasano et al., 2006). At 7 days post-treatment, Fasano et al. (2005) accounted for approximately 4-7% of total radioactivity administered in selected tissues in the rat. Similarly, we determined that after 120 hours, approximately 2-4% of 8-2 FTOH administered is present as the terminal metabolites, PFOA and PFNA. At these latter time-points in our study, the majority of terminal metabolites (approximately 2-3%) are retained in serum (Figure 4-2D).

Based on the proposed metabolic pathways reported by Martin et al. (2005) and expanded by Fasano et al. (2006), 8-2 FTOH is metabolized by either dehydrogenases or cytochrome P450s. In our study, we examined the concentrations of PFCs identified as key-branching points in 8-2 FTOH metabolism in rats (Martin et al., 2005; Fasano et al., 2006). β-oxidation is the major route of 8-2 FTOH metabolism in mice as evidenced by the concentrations of PFOA when compared to PFNA; however; α-oxidation is also occurring indicated by the presence of PFNA. Based on the concentrations of terminal metabolites detected following a single oral exposure to 8-2 FTOH in this study, exposure to precursor compounds could aid in explaining the accumulation of PFOA and PFNA in remote areas.

Acknowledgements

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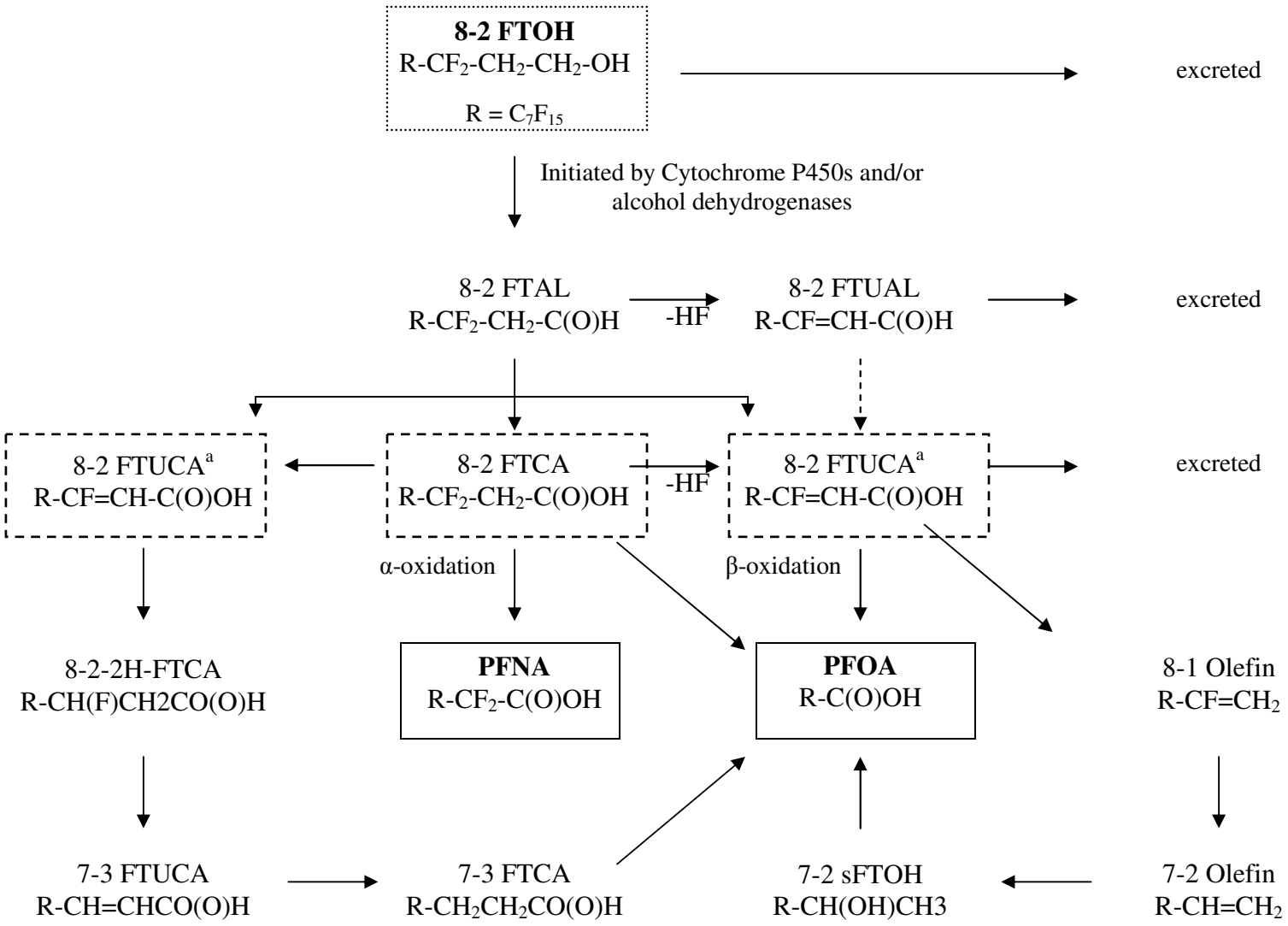

Figure 4-1: Compiled 8-2 FTOH metabolism scheme as based on *in vivo* and *in vitro* work of Hagen et al. (1985), Kudo et al. (2002), Martin et al. (2005), and Fasano et al. (2006). Compounds identified in this study include parent (8-2 FTOH; dotted box), intermediate (8-2 FTCA and 8-2 FTUCA; dashed box) and terminal metabolites (PFOA and PFNA; solid box).

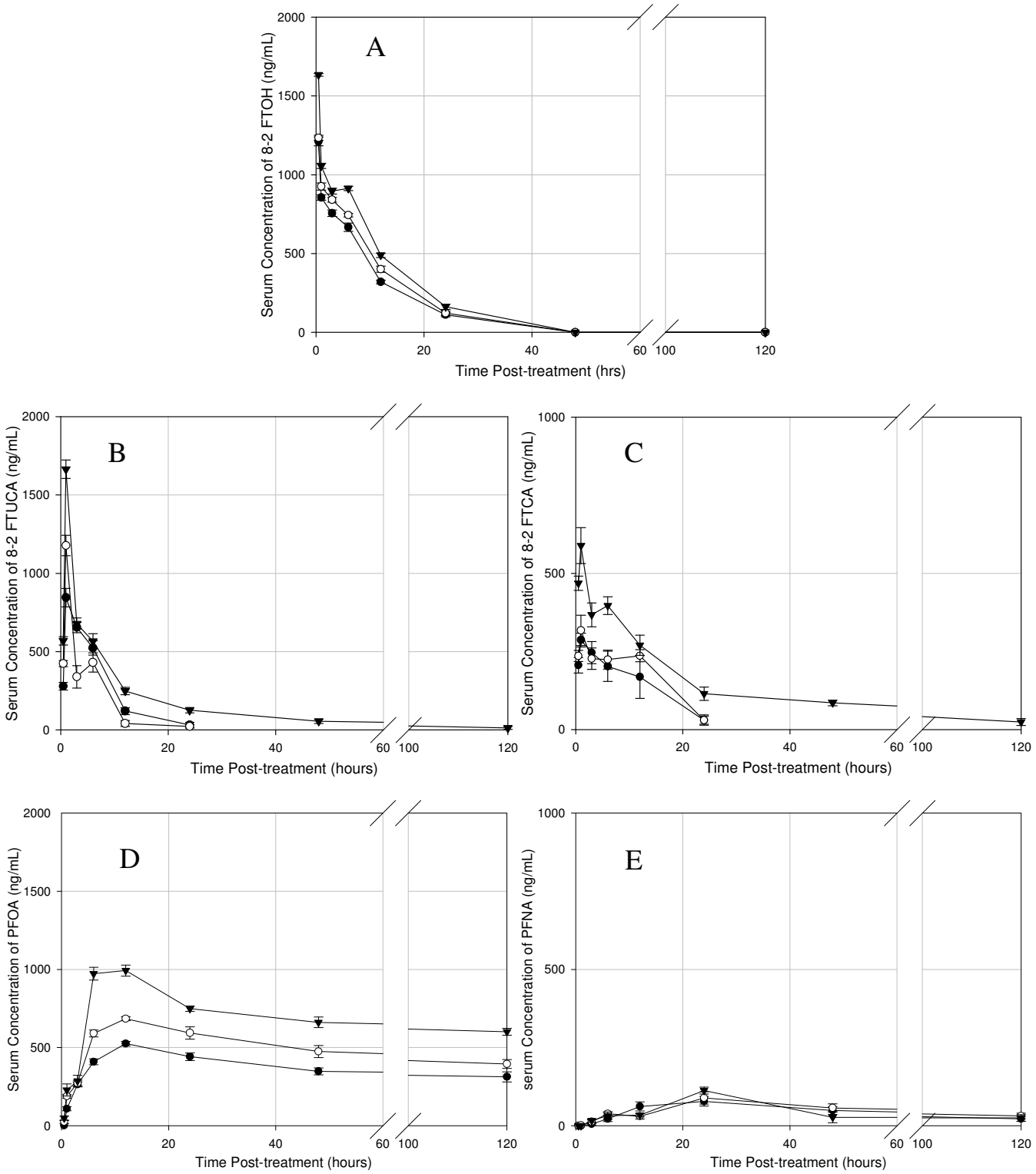

Figure 4-2: Serum concentrations of 8-2 FTOH (A), 8-2 FTUCA (B), 8-2 FTCA (C), PFOA (D), and PFNA (E) following a single oral exposure to 10 (●), 20 (○), or 30(▼) mg/kg BW 8-2 FTOH in CD-1 mice. In order to illustrate the temporal trend of terminal metabolite formation, each graph has been broken from 60-100 hours post-treatment.

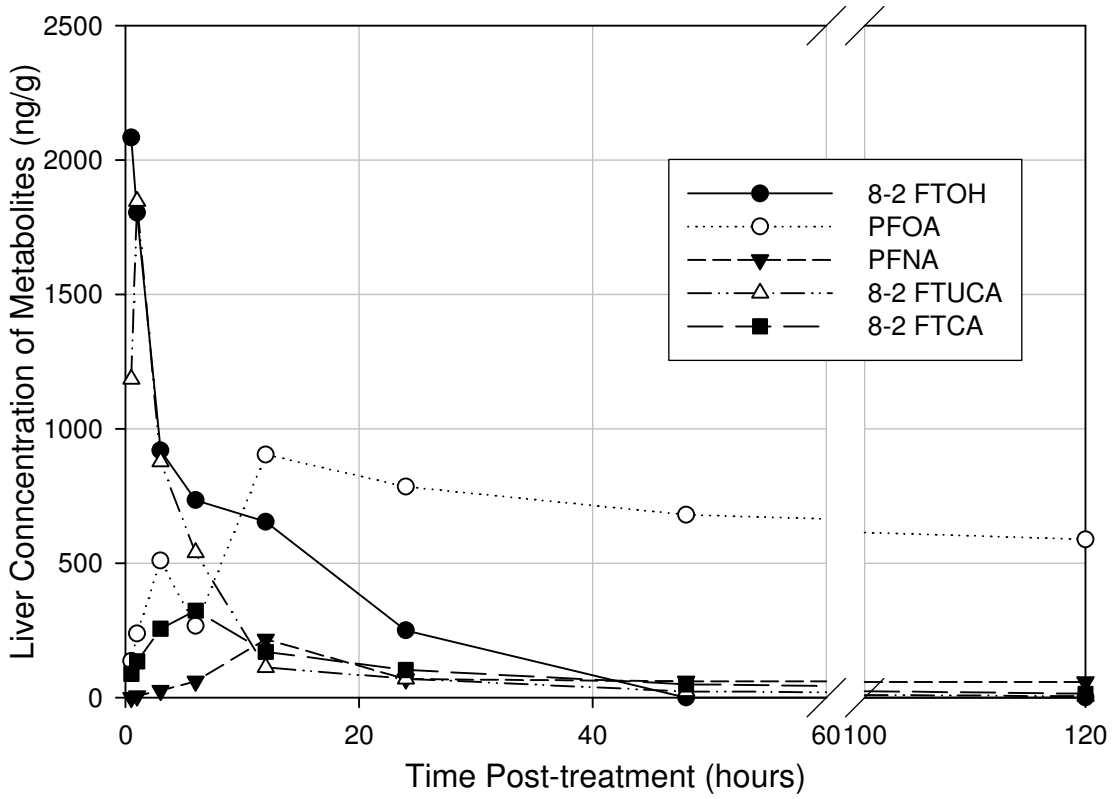

Figure 4-3: Parent, intermediate and terminal metabolites identified in liver following oral exposure to 30 mg 8-2 FTOH/kg BW in male CD-1 mice. PFOA (○) is the only metabolite detected after 48 hours post-treatment.

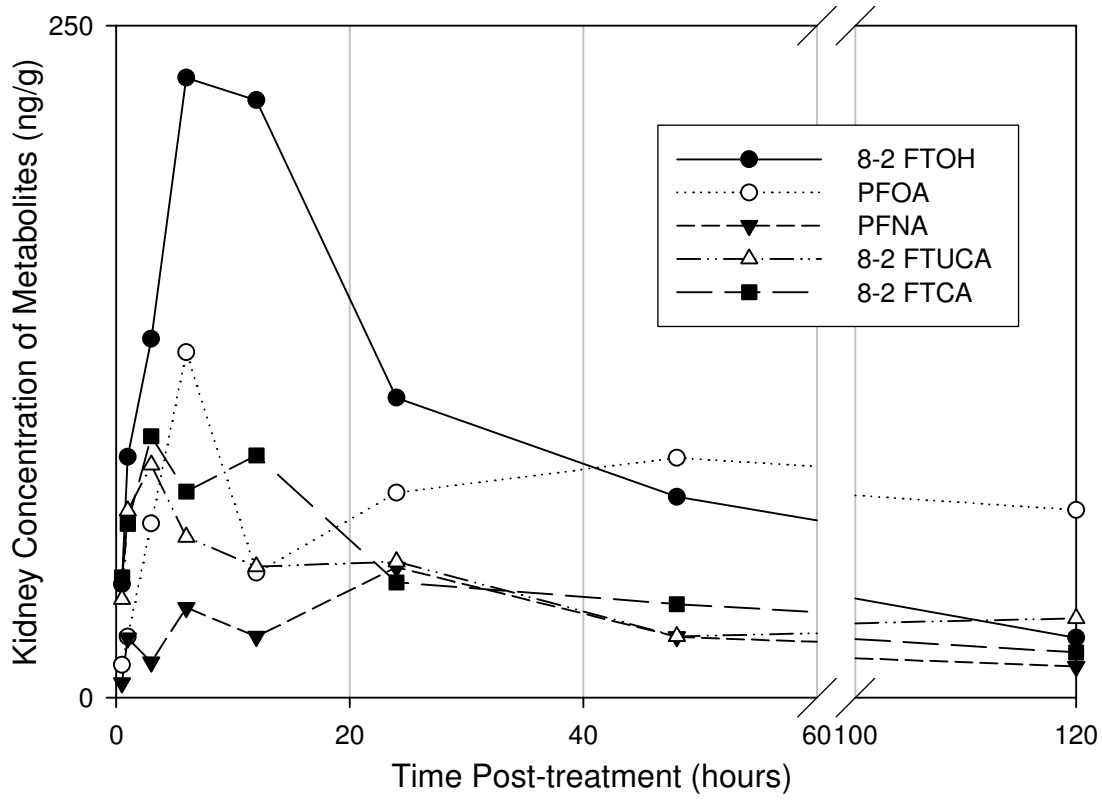

Figure 4-4: Representative distribution of 8-2 FTOH and metabolites identified kidneys following oral exposure to 30 mg 8-2 FTOH/kg BW in male CD-1 mice. Parent and metabolite distribution in other tissues investigated in this study (spleen, gonads and brain) as well as fat is provided in the supplemental data.

Table 4-1: Relevant perfluorinated chemical names, abbreviations, molecular structures and CAS numbers identified in and/or pertinent to the metabolism of 8-2 FTOH in male CD-1 mice.

Chemical Name	MW	CAS Number	Structural Formula	Abbreviation
8-2 fluorotelomer alcohol	464	678-39-7	$CF_3(CF_2)_7CH_2CH_2OH$	8-2 FTOH
8-2 fluorotelomer aldehyde	462	NF[a]	$CF_3(CF_2)_7CH_2C(=O)H$	8-2 FTAL
8-2 fluorotelomer unsaturated aldehyde	442	NF[a]	$CF_3(CF_2)_6C(F)=CHC(=O)H$	8-2 FTUAL
8-2 fluorotelomer acid	478	27854-31-5	$CF_3(CF_2)_7CH_2COOH$	8-2 FTCA
8-2 fluorotelomer unsaturated acid	458	70887-84-2	$CF_3(CF_2)_6C(F)=CHCOOH$	8-2 FTUCA
7-3 fluorotelomer acid	442	812-70-4	$CF_3(CF_2)_6CH_2CH_2COOH$	7-3 FTCA
7-2 fluorotelomer unsaturated acid	440	755-03-3	$CF_3(CF_2)_6C(H)=CHCOOH$	7-3 FTUCA
perfluorooctanoic acid	414	335-67-1	$CF_3(CF_2)_6COOH$	PFOA
perfluorononanoic acid	464	375-95-1	$CF_3(CF_2)_7COOH$	PFNA

[a] NF=not found. A CAS number was unavailable for this compound.

Table 4-2: Serum kinetic parameters for parent, intermediate and terminal metabolites following a single oral exposure to 30 mg 8-2 FTOH/kg BW in male CD-1 mice.

Dose	Parameter	8-2 FTOH	8-2 FTUCA	8-2 FTCA	PFOA	PFNA
30 mg/kg	$t_{1/2}$ (hrs)[a]	10.1 (6.2[b])	16.0	14.5	177.1	64.8
	k_{el} (1/hr)	8.3×10^{-2}	1.0	6.5×10^{-3}	3.7×10^{-3}	1.2×10^{-2}
	$t_{max(obs)}$ (hrs)	0.5	1	1	12	24
	C_{max} (ng/mL)	1634 ± 44	1665 ± 67	588 ± 42	992 ± 38	113 ± 18
	AUC_{obs} (ng·hr/mL)	1.54×10^4	1.41×10^4	1.30×10^4	8.12×10^4	4.71×10^3
	AUC_{∞} (ng·hr/mL)	1.62×10^4	1.43×10^4	8.56×10^3	2.44×10^5	6.7×10^3

[a] Calculated $t_{1/2}$ based on linear, least-squares regression.
[b] Calculated $t_{1/2}$ based on least-squares regression following log-transformation of the data.

Table 4-3: Percent of total dose accounted for as 8-2 FTOH, 8-2 FTUCA, 8-2 FTCA, PFOA, and PFNA over time in male CD-1 mice following a single oral exposure to 30 mg 8-2 FTOH/kg BW.

Time Post-Treatment (hours)	Percent Total Dose							
	Serum	**Liver**	**Kidney**	**Gonads**	**Brain[a]**	**Fat[a]**	**Spleen**	**TOTAL:**
0.5	4.4	7.3	0.08	0.04	0.01	0.14	ND[b]	**11.9**
1	5.7	8.4	0.15	0.06	0.02	0.41	0.01	**14.8**
3	3.6	5.5	0.22	0.09	0.04	0.50	0.02	**10.0**
6	4.8	4.0	0.30	0.11	0.05	0.36	0.04	**9.7**
12	3.4	4.5	0.24	0.07	0.03	0.30	0.03	**8.6**
24	2.2	2.8	0.19	0.05	0.02	0.27	0.02	**5.5**
48	1.5	1.8	0.14	0.04	0.02	0.19	0.02	**3.7**
120	1.2	1.5	0.09	0.04	0.01	ND	0.01	**2.8**

[a] Brain and fat were only analyzed for 8-2 FTOH due to preliminary experiments in which intermediate and terminal metabolites were not identified in these tissues.
[b] ND=not determined at this time-point.

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Supplemental Data

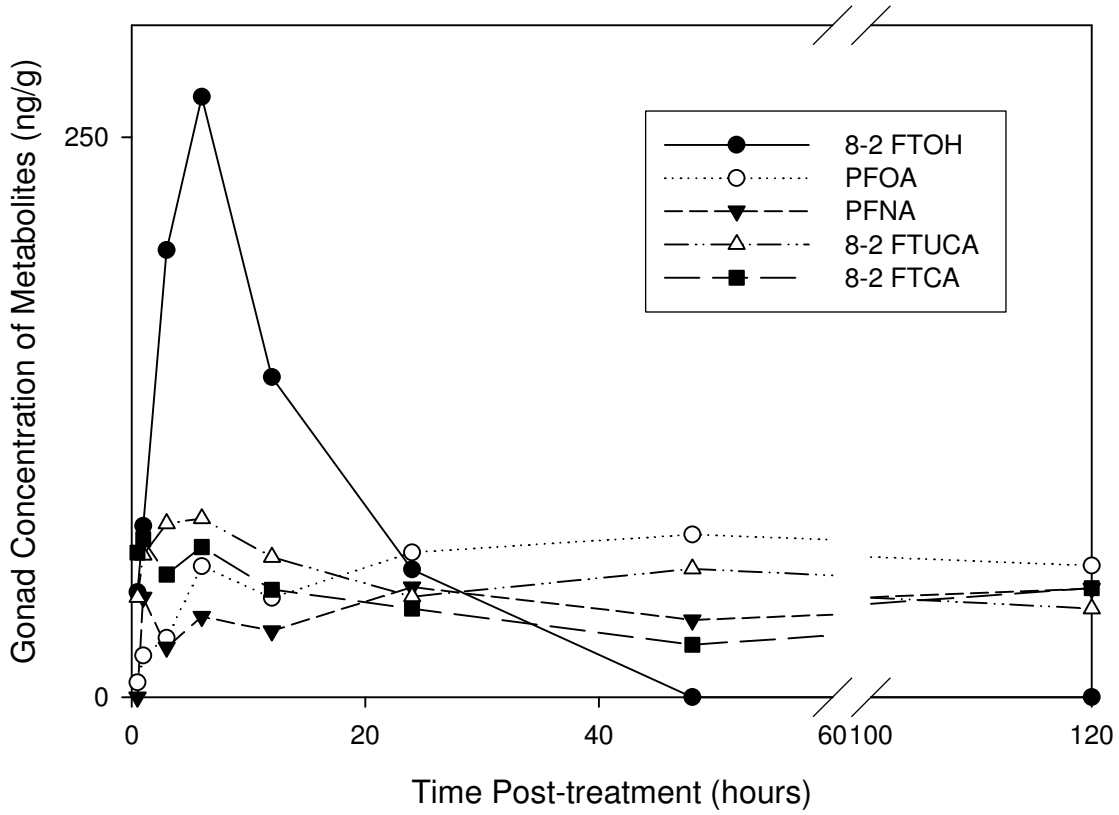

Figure 4-1S: Parent, intermediate and terminal metabolites identified in gonads following oral exposure to 30 mg 8-2 FTOH/kg BW in male CD-1 mice.

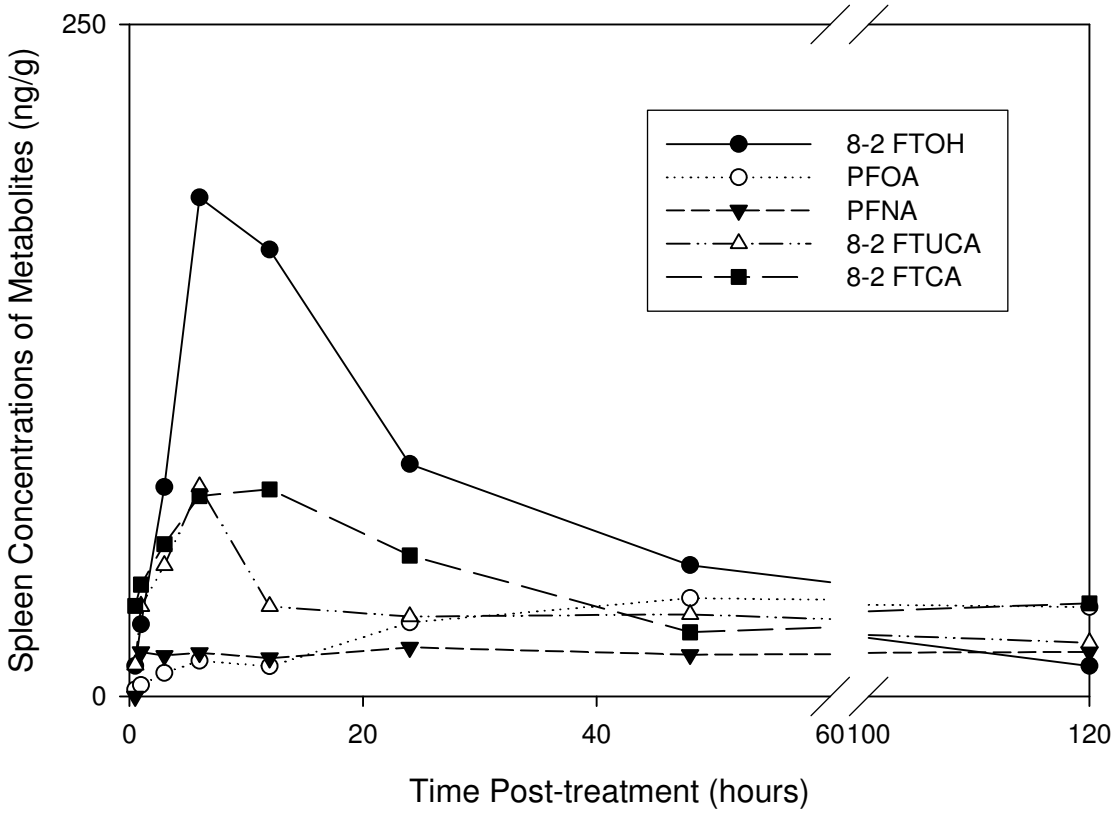

Figure 4-2S: Parent, intermediate and terminal metabolites identified in spleen following oral exposure to 30 mg 8-2 FTOH/kg BW in male CD-1 mice.

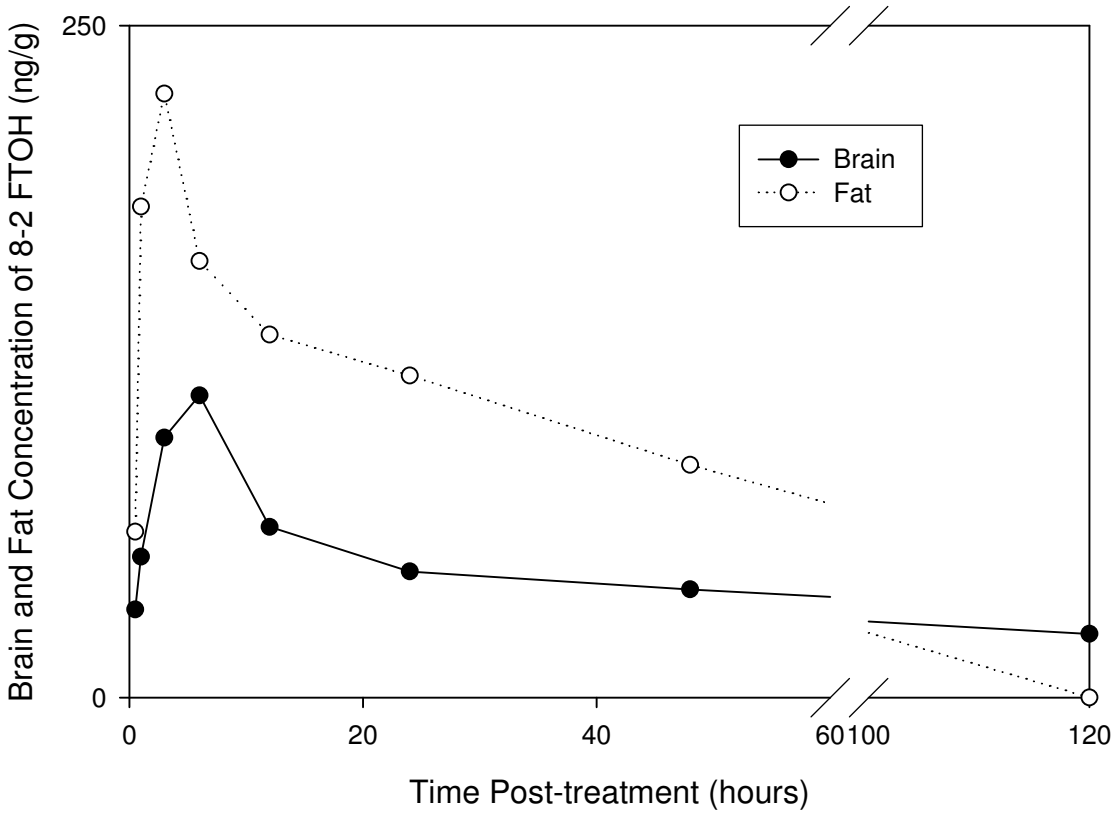

Figure 4-3S: 8-2 FTOH in brain (●) and fat (○) following oral exposure to 30 mg 8-2 FTOH/kg BW in male CD-1 mice.

Table 4-1S: Serum kinetic parameters for parent, intermediate and terminal metabolites following a single oral exposure either 10 or 20 mg 8-2 FTOH/kg BW in male CD-1 mice.

Dose	Parameter	8-2 FTOH	8-2 FTUCA	8-2 FTCA	PFOA	PFNA
10 mg/kg	$t_{1/2}$ (hrs)[a]	12.6 (6.68[b])	12.2	13.6	155.0 (6.5 d)	74.6 (3.1 d)
	k_{el} (1/hr)	9.6×10^{-2}	1.4	9.6×10^{2}	4.3×10^{-3}	1.3×10^{-2}
	$t_{max(obs)}$ (hrs)	0.5	1	1	12	24
	C_{max} (ng/mL)	1211 ± 36	844 ± 29	286 ± 24	525 ± 33	79 ± 18
	AUC_{obs} (ng·hr/mL)	1.11×10^{4}	6.77×10^{3}	3.99×10^{3}	4.33×10^{4}	5.21×10^{3}
	AUC_{∞} (ng·hr/mL)	1.15×10^{4}	6.67×10^{3}	4.00×10^{3}	1.16×10^{5}	7.0×10^{3}
20 mg/kg	$t_{1/2}$ (hrs)	12.1 (7.90[b])	15.8	14.2	142.5 (5.9 d)	83.3 (3.5 d)
	k_{el} (1/hr)	9.3×10^{-2}	1.6	9.5×10^{-2}	4.7×10^{-3}	1.0×10^{-2}
	$t_{max(obs)}$ (hrs)	0.5	1	1	12	24
	C_{max} (ng/mL)	1234 ± 42	1177 ± 66	317 ± 26	684 ± 32	89 ± 12
	AUC_{obs} (ng·hr/mL)	1.27×10^{4}	5.14×10^{3}	4.69×10^{3}	5.74×10^{4}	5.93×10^{3}
	AUC_{∞} (ng·hr/mL)	1.31×10^{4}	5.11×10^{3}	4.71×10^{3}	1.41×10^{5}	8.9×10^{3}

[a] Calculated $t_{1/2}$ based on linear, least-squares regression.
[b] Calculated $t_{1/2}$ based on least-squares regression following log-transformation of the data.

Table 4-2S: Total percent dose accounted for as 8-2 FTOH, 8-2 FTUCA, 8-2 FTCA, PFOA, and PFNA over time in male CD-1 mice following a single oral exposure either 10 or 20 mg 8-2 FTOH/kg BW.

Time Post-Treatment (hours)	Percent Total Dose							
	Serum	**Liver**	**Kidney**	**Gonads**	**Brain[a]**	**Fat[a]**	**Spleen**	**TOTAL:**
				10 mg/kg				
0.5	8.1	17.2	0.04	0.03	0.02	0.96	0.01	**26.4**
1	10.2	14.6	0.12	0.03	0.06	0.74	0.02	**25.7**
3	9.4	11.4	0.31	0.11	0.08	0.83	0.02	**22.2**
6	9.0	7.9	0.59	0.18	0.07	0.44	0.08	**18.3**
12	6.0	6.9	0.21	0.13	0.05	0.51	0.07	**13.9**
24	3.6	3.6	0.32	0.12	0.05	0.23	0.03	**7.9**
48	2.1	2.4	0.15	0.09	0.04	ND[b]	0.03	**4.8**
120	1.8	2.0	0.14	0.08	0.02	ND[b]	0.02	**4.1**
				20 mg/kg				
0.5	4.6	9.3	0.52	0.02	0.02	0.36	ND[b]	**14.9**
1	6.3	9.6	0.42	0.06	0.03	0.54	0.01	**17.0**
3	4.1	5.1	0.46	0.05	0.05	0.49	0.01	**10.3**
6	5.0	4.6	0.42	0.06	0.05	0.23	0.04	**10.4**
12	3.5	4.8	0.16	0.04	0.03	0.23	0.01	**8.9**
24	2.2	2.4	0.12	0.04	0.03	0.15	0.01	**5.0**
48	1.4	1.7	0.04	0.05	0.05	ND[b]	0.01	**3.3**
120	1.1	1.6	0.05	0.05	0.02	ND[b]	0.01	**2.9**

[a] Brain and fat were only analyzed for 8-2 FTOH due to preliminary experiments in which intermediate and terminal metabolites were not identified in these tissues.
[b] ND=not determined at this time-point.

CHAPTER 5

PERFLUOROOCTANOIC ACID (PFOA) AND PERFLUORONONANOIC ACID (PFNA) IN FETAL AND NEONATAL MICE FOLLOWING *IN UTERO* EXPOSURE TO 8-2 FLUOROTELOMER ALCOHOL (FTOH)[3]

[3] Henderson, W. Matthew and Mary Alice Smith. 2006. *Toxicological Sciences. Available online 8 November 2006,* 10.1093/toxsci/kfl162.

Abstract

8-2 fluorotelomer alcohol (FTOH) and its metabolites, perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA), are developmental toxicants, but metabolism and distribution during pregnancy is not known. To examine this, timed-pregnant mice received a single gavage dose (30 mg 8-2 FTOH/kg BW) on gestational day (GD) 8. Maternal and neonatal serum and liver as well as fetal and neonatal homogenate extracts were analyzed using GC/MS. During gestation (GD9 to GD18), maternal serum and liver concentrations of PFOA decreased from 789±41 to 668±23 ng/mL and from 673±23 to 587±55 ng/g, respectively. PFOA was transferred to the developing fetuses as early as 24 hours post-treatment with concentrations increasing from 45±9 ng/g (GD10) to 140±32 ng/g (GD18), while PFNA was quantifiable only at GD18 (31±4 ng/g). Post-partum, maternal serum PFOA concentrations decreased from 451±21 ng/mL post-natal day (PND) 1 to 52±19 ng/mL (PND15) and PFNA concentrations, although 5-fold less, exhibited a similar trend. Immediately after birth, pups were cross-fostered with dams that had been treated during gestation with 8-2 FTOH (T) or vehicle (C) resulting in four treatment groups in which the first letter represents *in utero* (fetal) exposure and the second represents lactational (neonatal) exposure: C/C, T/C, C/T, T/T. On PND1, neonatal whole-body homogenate concentrations of PFOA from T/T and T/C groups averaged 200±26 ng/g, decreased to 149±19 ng/g at PND3 and this decreasing trend was seen in both neonatal liver and serum from PND3 to PND15. Based on detectible amounts of PFOA in neonatal serum in the C/T group on PND3 (57±11 ng/mL) and on PND15 (58±3 ng/mL), we suggest the neonates were exposed through lactation. In conclusion, exposure of neonates to PFOA and PFNA occur both pre- and post-natally following maternal 8-2 FTOH exposure on GD8.

Introduction

The fluorotelomer alcohols (FTOHs) are used in the manufacture of fluorinated surfactants and polymers and in the surface-active modification of consumer products. They are linear chain polyfluorinated alcohols in which the –OH moiety links the polyfluorinated alkyl tail to various polymers via ester, amide, urethane, and ether linkages. Furthermore, the FTOHs are used as intermediates in the synthesis of dyes, paints, adhesives, polymers, and waxes (Dinglasan-Panlilio and Mabury, 2006). The FTOHs have been shown to be present in the North American troposphere at concentrations up to 165 pg/m^3 (Stock et al., 2004) and it is estimated that the global production from 2000 to 2002 exceeded 5000 tons/year (Prevedouros et al., 2006).

Following release, 8-2 FTOH and structurally analogous compounds are likely transported via atmospheric processes and subsequently transformed into PFOA via both metabolism and biodegradation processes (Fasano et al., 2006, Kudo et al., 2005, Martin et al., 2005). Perfluorinated carboxylic acids (PFCAs) are formed during atmospheric oxidation of FTOHs (Ellis et al., 2004, Wallington et al., 2006). PFOA was formed by aerobic degradation of 8-2 FTOH in a mixed microbial system (Dinglasan et al., 2004, Wang et al., 2005), and via mammalian metabolism of these precursor compounds (Fasano et al., 2006, Kudo et al., 2005, Martin et al., 2005). However, PFCAs including PFOA and perfluorononanoic acid (PFNA) are resistant to metabolism and environmental biodegradation reactions including oxidation, hydrolysis, and reductive halogenation.

Both PFOA and PFNA are of particular concern because they are ubiquitous in both human and environmental matrices (Martin et al., 2002, Olsen et al., 2003, Stock et al., 2004, Taniyasu et al., 2005), have been associated with reproductive and developmental toxicity in

laboratory animals (Butenhoff et al., 2004a, Lau et al., 2004, Lau et al., 2006), and human exposure has been demonstrated (Emmett et al., 2006, Olsen et al., 2004a, Olsen et al., 2004b, Olsen et al., 2005).

PFOA toxicity studies have been conducted in monkeys, rabbits, mice and rats (Butenhoff, et al., 2004b, Kennedy et al., 2004, Kudo and Kawashima, 2003, Lau et al., 2004) and epidemiological investigations have been conducted in humans (Olsen et al., 2000, Emmett et al., 2006, Olsen et al., 2004a, Olsen et al., 2004b, Olsen et al., 2005). Although these studies show that pharmacokinetics of the fluorinated compounds vary between species, PFOA has been shown to bioaccumulate in higher trophic organisms including humans (Ehresman et al., 2006, Smithwick et al., 2006, Tomy et al., 2004). Although PFOA's biomagnification potential is less than that of the structurally-related perfluorooctane sulfonate (PFOS), bioaccumulation was shown to occur in an aquatic marine food web (Tomy et al., 2004). Furthermore, numerous perfluorinated chemicals (PFCs) such as PFOS (Lau et al., 2004, Lau et al., 2003, Luebker et al., 2005a, Luebker et al., 2005b, Thibodeaux et al., 2003), PFCAs including PFOA (Butenhoff et al., 2004a) and perfluorodecanoic acid (PFDA: Harris and Birnbaum, 1989; Olson and Andersen, 1983) adversely affect both pre- and post-natal development as well as cause neurological and endocrine deficits in laboratory animals (Johansson et al., 2006).

PFOS is developmentally toxic in rats (Butenhoff et al, 2004a; Grasty et. al., 2005, Thibodeaux et al., 2003) and PFOA is developmentally toxic in mice (Lau et al., 2004, Lau et al., 2006) resulting in embryonic and post-natal death and growth retardation when animals are administered at least 10 mg PFOS or PFOA/kg/day over total gestation. Interestingly, it is suggested that female rats are unlike humans in their ability to rapidly excrete PFOA (Kudo et al., 2002, Kudo et al., 2001). It is hypothesized that rats possess an organic acid transport

(OAT)-mediated excretion process that rapidly and efficiently decreases the body burden of PFOA (Kudo et al., 2002) as well as other perfluorinated chemicals. These processes are not maximally expressed until sexual maturity in rats (Buist et al., 2002, Hinderliter et al., 2006). Although the OAT transporters are highly conserved across species it has been shown that modulation and transcription of various isoforms (namely OAT2) are age- and sex-dependent in rats but less so in mice (Buist et al. 2002, Buist and Klaasen, 2004). In comparison, in the human kidney, OAT2 was the most abundant transcript detected (Bahn et al., 2004); however, gender-based differences in humans have not been definitively elucidated. Based on available data, male (Buist et al., 2002; Hinderliter et al., 2006, Kudo et al., 2002) and female (Lau et al., 2003) rats do not appear to be an appropriate animals model for understanding the human health risk of PFOA exposure (Harada et al., 2005, Calafat et al., 2006).

More recently, the effects of PFOA exposure during pregnancy were investigated in the mouse. PFOA produced dose-dependent effects on the number of resorptions, maternal and fetal weight gain, post-natal survival and growth deficits at doses between 10-30 mg PFOA/kg/day with full litter loss occurring at 40 mg PFOA/kg/day (Lau et al., 2006). Early gestational exposure has been associated with growth reduction, low body weight, and poor post-natal survival of pups (Lau et al., 2006). Lau et al. (2006) concluded that *in utero* exposure to PFOA provides the major contribution of the associated adverse effects and lactational exposure may be a minor contributor to developmental outcomes. Hinderliter et al. (2005) observed that following gestational exposure, PFOA milk concentrations were proportional to maternal administered dose and thus neonates received a constant dose from the dam until weaning as a result of lactational transport. Secondly, upon repeated dosing with PFOA, approximately 8% of the total administered maternal dose in rats is transferred to the developing neonate via lactation

(Hinderliter et al., 2005). Furthermore, neonatal PFOA exposure results in developmental neurotoxic effects. Both PFOA and PFDA adversely affect the cholinergic system in adult mice after neonatal exposure as evidenced by deficits in locomotion and total activity in adult mice, similar to other persistent organic pollutants (Johansson et al., 2006).

Information on the metabolism of 8-2 FTOH in mice is limited and there are no published studies elucidating the maternal-fetal and neonatal distribution of 8-2 FTOH and its oxidation metabolites, PFOA and PFNA. In rats, 8-2 FTOH increases fetal skeletal variations at 500 mg/kg/d with a no observed adverse effect level defined as 200 mg/kg/d (Mylchreest et al., 2005) and behaves like xenoestrogens *in vitro* (Maras et al., 2006). In order to elucidate plausible exposure scenarios, the objective of this study was to investigate the maternal-fetal transfer of 8-2 FTOH and its metabolites, PFOA and PFNA, as well as the role of lactational transfer and subsequent metabolism of these metabolites in mouse neonates exposed *in utero* or post-natally (by lactation).

Materials and Methods

Animals: Timed-pregnant CD-1 mice from Charles River Laboratories (Raleigh, NC) were received on gestational day (GD) 5 and allowed to acclimate for 3 days prior to treatment. Pregnant mice were housed in micro-isolator cages and the rooms maintained between 21º-26ºC and 40-70% relative humidity with a 12 hour light/dark cycle. Animals were fed Lab Diet Certified Rodent Chow with both food and water provided *ad libitum*. All animal studies were conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* (1996) and were approved by the University of Georgia's IACUC committee. Mice received a gavage dose of 30 mg 8-2 FTOH/kg body weight (BW) in a

propylene glycol/water (1:1) vehicle or vehicle control on GD8. The dosing solution was repeatedly homogenized with a hand-held Tissue-Tearor™ (Biospec Products, Inc., Bartlesville, OK) throughout the dosing period.

Gestational Experimental Design: For the *in utero* exposure study, mice (n=41) were divided into two groups, control (n=15) and treated (n=26). Animals were serially sacrificed at 1 (GD9), 2 (GD10), 5 (GD13), 7 (GD15) and 10 (GD18) days post-treatment. Dams were sacrificed by CO_2 asphyxiation, maternal serum and liver samples were collected and the fetuses were removed from the uterus.

Cross-Fostering Experimental Design: In the cross-foster study, timed-pregnant mice were divided into control (n=34) and treated groups (n=36) and similarly treated with either vehicle or 30 mg/kg BW 8-2 FTOH in vehicle on GD8. Following parturition (post-natal day (PND) 0), half of the treated litters were cross-fostered with dams treated with vehicle control resulting in four treatment groups: no exposure (C/C), *in utero* exposure only (T/C), post-natal exposure only (via lactation) (C/T), and both *in utero* and post-natal (lactational) exposure (T/T). These animals were sacrificed on PND 1, 3, and 15, and samples were collected for PFC analysis. The schematic in Figure 1 illustrates the design of both the *in utero* and post-natal cross-foster study.

Sera and Tissue Collection: Blood was collected via cardiac puncture and placed in 1.2 mL centrifuge tubes on ice prior to centrifugation at 2500 rpm (608 x g) for 15 minutes. Serum was transferred to a new tube and was stored at -80ºC prior to fluorochemical quantification. Maternal liver as well as placental and fetal tissues were excised for extraction and PFC analysis by GC/MS. Neonatal serum and liver ($n \geq 5$ per dam) was collected on PND3 and PND15. All

tissues were weighed on a Mettler PM4000 balance and immediately flash frozen with a combination of liquid nitrogen and dry ice and stored at -80ºC until analysis.

Chemicals: All chemical reagents used in this study were obtained at the highest purity, greater than 98% as determined by the supplier. 8-2 fluorotelomer alcohol, (8-2 FTOH; CAS Number 678-39-7) was purchased from Oakwood Research Chemicals (West Columbia, SC) as were all other PFCs and their methyl ester counter parts used for comparative analysis. The PFCs used in this study, along with their CAS numbers and structural formulas are shown in Table 5-1. Tetrabutylammonium hydrogen sulfate (TBAHS) and sodium carbonate were purchased from Aldrich Chemical (Milwaukee WI), as were Diazald®, carbitol, and potassium hydroxide.

PFC Analysis: Both alcohol and non-volatile acid analyses were conducted via gas chromatography coupled with mass spectrometry (GC/MS) using a previously published method (Henderson et al., 2006). Briefly, samples (250 uL serum, 1.0 g liver or 0.5-1.0 g fetus/neonate) were homogenized in equal volumes of phosphate-buffered saline and extracted following the method of Hansen et al. (2001). After extraction, carboxylic and telomer acids in the extracts were derivatized with diazomethane (Ngan and Toofan, 1991) and analyzed using a HP 6890 Series Gas Chromatograph equipped with a 5973 Mass Selective Detector and a HP 6890 Series Injector (Palo Alto, CA). The mass spectrometer was operated with the electron ionization (EI) source in selected ion monitoring (SIM) mode at *m/z* values 131, 169, 231, and 331 (Henderson et al., 2006). Furthermore, all data points were confirmed and quantified with LC/MS-MS using the method of Powley and co-workers (2005), incorporating an internal isotopic standard for intra-laboratory comparison. All samples were extracted and derivatized on ice and remained

capped throughout analysis to prevent the possible evaporative loss of 8-2 FTOH and other volatile intermediates.

Statistical Analysis: Although the gestational and cross-fostering studies were conducted in independent studies, when appropriate, data was combined for statistical analysis such as maternal and fetal/neonatal weight gain. For comparison between control and treated dams, averages ($n \geq 5$) were analyzed with a general linear model (GLM) and further significant differences were analyzed with a Students t-test. All statistical analysis was conducted with Statistical Analysis Software (SAS) (Cary, NC) and a p-value of 0.05 was used as the level of desired significance.

Results

After treatment with 8-2 FTOH or vehicle, no obvious signs of toxicity such as lethargy or morbidity were observed in pregnant mice. Both control and treated mice gained approximately 25 grams during gestation, and there were no significant differences in weight gain between treatment and control groups throughout the study (Figure 5-2). Similarly, fetal and neonatal body weights were not affected by treatment with 8-2 FTOH (Tables 5-2 and 5-3).

Average litter size throughout the gestational study was not significantly different between control and treated groups (Table 5-4). Similarly, average litter size was unaffected by treatment in the cross-foster study with averages for control and treated groups at 13±2 neonates. Litters were examined on GD9, 10, 13, 15, and 18 and no significant differences in implantations, resorptions or fetal deaths were observed in the gestational experiment. Fetuses were examined for gross malformations at GD15 and GD18 in which two non-viable fetuses were excised from 8-2 FTOH treated dams (Table 5-4). However, in the cross-fostering

experiment, 31% of treated dams had at least one non-viable neonate and 27% of the nonviable neonates were characterized as having anencephaly or exencephaly (Table 5-4). It should be noted that one neonate with anencephaly was viable immediately following birth, but was euthanized and is included in the non-viable group. Among viable neonates at term, there were no significant post-natal losses up to PND15.

Although there were no apparent treatment-related effects on maternal body weight (BW) gain, 8-2 FTOH treatment did result in significant increases in the maternal liver weight (LW) to body weight ratio (relative liver weight; LW:BW) as well as a transient increase in absolute liver weight at GD18 (Table 5-2). Maternal LW:BW was significantly elevated in the 8-2 FTOH treated groups at GD13 thru GD18 (Table 5-2) and this effect continued throughout the post-natal period examined (PND1 thru PND15) (Table 5-3). In comparison, significant increases in LW:BW ratio were seen at both PND5 and PND15 in neonates exposed *in utero* (T/T and T/C). Furthermore, neonatal absolute liver weights were significantly elevated in group T/T at the same time-points (Table 5-3). Due to limitations in liver size and amount of sample needed for PFC analysis, neonatal livers were not excised from PND1 neonates.

When examining the distribution and metabolism in maternal serum, 8-2 FTOH was no longer detectable in maternal serum or liver 24 hours after treatment (GD9). PFOA and PFNA were the only metabolites detected in samples analyzed for 8-2 FTOH, 8-2 fluorotelomer acid (8-2 FTCA), 8-2 fluorotelomer α,β-unsaturated acid (8-2 FTUCA), PFOA and PFNA, all commercially available intermediate metabolites (Figure 5-3). Furthermore, no parent compound (8-2 FTOH) was detected in fetal or neonatal tissues at 24 hours or throughout the study. Placental concentrations of both PFOA and PFNA were at maximal concentrations 24 hours post-treatment and decreased throughout gestation. Placentas from GD18 had a PFOA

concentration of 49 ±13 ng/g, approximately 10-fold less than those determined in serum and liver samples (Figures 5-3 and 5-4).

In both the gestational and cross-fostering studies, PFOA was the most abundant metabolite in all tissues examined including those from dams, fetuses, and neonates (Figures 5-3 to 5-5 and Table 5-5). Following *in utero* exposure to 8-2 FTOH, fetal concentrations of both PFOA and PFNA increase temporally reaching maximal concentrations at or near parturition (Figure 5-5). Neonatal body burdens of PFOA and PFNA decrease temporally following parturition up to PND15 (Table 5-5). PFOA and PFNA are approximately 1.5 times more abundant in the serum of neonatal mice compared to liver concentrations based on percent BW (Table 5-5). Furthermore, evidence for the lactational transport of both PFOA and PFNA is illustrated by the presence of PFOA and PFNA in groups exposed to treated dams only during lactation and elevated concentrations in the group exposed to treated dams both *in utero* and post-natally when compared to T/C groups (Table 5-5).

During gestation, maternal serum concentrations of PFOA were highest at GD9 and decreased temporally up to GD18 (Figure 5-3). Similarly, maternal PFNA concentrations were at maximal levels one day post-treatment (GD9) and decreased 3-fold prior to parturition (Figure 5-3). In maternal liver at GD18, concentrations of PFOA were approximately 6 times greater than concentrations of PFNA. Both maternal liver and serum concentrations of PFNA were less than that of PFOA; however, the two metabolites decreased similarly throughout gestation. PFOA and PFNA were transferred to the developing fetuses as early as 24 hours post-treatment (GD9) with PFOA concentrations increasing from GD9 to GD18 (Figure 5-5). In fetuses, PFNA was above method quantification limits only in the GD18 group, but the overall tissue distribution was similar to PFOA (Figure 5-5).

In the cross-fostering study, maternal serum concentrations of PFOA decreased temporally from PND1 up to PND15 in 8-2 FTOH treated animals. PFNA concentrations, although less, exhibited a similar trend reaching concentrations below method detection limits at or before PND15 (Table 5-5). Maternal liver concentrations of PFOA were highest in the cross-fostering experiment immediately following parturition having reached maximal levels during gestation. These concentrations temporally decreased to the lowest concentrations detected at PND15 (Figure 5-3). No statistical differences in PFOA concentrations in serum or liver were present between control dams (C/C) or those that received *in utero*-treated pups (T/C). On PND1, neonatal tissue concentrations of PFOA from *in utero* treated groups (T/C and T/T) were not statistically different and concentrations had decreased by PND3, most notably in the group lacking postnatal (ie. lactational) exposure (T/C, Table 5-5). This trend continued in both neonatal serum and livers from T/T and T/C groups as PFOA and PFNA concentrations temporally decreased from PND3 to PND15 (Table 5-5). Neonates appear to be exposed through lactation based on detectible amounts of PFOA in both neonatal serum and liver on PND3 and PND15 in the group (C/T) exposed during the neonatal period only. Throughout the study neither PFOA nor PFNA or any other metabolites were not detected in the control dams, fetuses or neonates at any time-point.

Discussion

A major concern of PFCs is their universal detection in mammalian tissues, not only from humans, but also from animals such as polar bears living in remote areas (Olsen et al., 2003, Smithwick et al., 2006). This suggests that atmospheric transport of volatile precursor compounds, such as 8-2 FTOH, may play a role in the remote distribution of the stable non-

volatile acids, PFOA and PFNA. Limited information is currently available on the metabolism of 8-2 FTOH in mammals. In this study *in utero* exposure to 8-2 FTOH in mice results in the formation of perfluorinated carboxylic acids, and the distribution of these metabolites to both the fetus and neonate affects growth and development, as illustrated by significant increases in relative liver weight (Table 5-3), post-partum mortality, and increase in neural tube defects in neonates (Table 5-4).

In vitro metabolism of 8-2 FTOH has been shown to be rapid with parent compound disappearing (78%) by 4 hours post-treatment (Martin et al., 2005) while Kudo et al. (2005) illustrated that intermediate metabolites reach peak concentrations at or near 6 hours post-treatment *in vivo*. In the present study, by 24 hours post-treatment, no parent compound or intermediate metabolites were identified in maternal or fetal samples. Only the terminal metabolites of 8-2 FTOH metabolism, PFOA and PFNA, were detected in maternal, fetal and neonatal tissues throughout the duration of this study (GD9 through PND15) (Figures 5-3 to 5-5 and Table 5-5).

PFOA is a developmental toxicant in both rats (Staples et al., 1985) and mice (Lau et al., 2006) but has not been shown to negatively affect reproductive function in rats (Butenhoff et al., 2004a). Furthermore, following chronic oral exposure during pregnancy in rats, PFOA has been detected in fetuses and in neonates confirming placental transfer (Hinderliter et al., 2005, Lau et al., 2006). Because no 8-2 FTOH was detected in fetal or placental tissues as early as 24 hours post-treatment, we suggest that the majority of 8-2 FTOH transformation is dependent on maternal metabolism. Our study shows that the maternal metabolism of 8-2 FTOH results in the fetal accumulation of PFOA (GD8-18). Similarly to Lau et al. (2006), we also saw an increase in neonatal mortality and based on Lau et al.'s findings, it is likely due to increasing PFOA

concentrations in the developing fetus. However, in contrast, neural tube defects (NTDs) were seen following *in utero* treatment with 8-2 FTOH. This may result from proximal transport of parent or intermediate metabolites (ie. 8-2 FTAL or 8-2 FTCA) and subsequent exposure to the developing fetus. More research is needed to elucidate the mechanisms of 8-2 FTOH developmental toxicity

Following 8-2 FTOH administration and subsequent metabolism, PFOA accumulates in the fetal compartment reaching maximal concentrations at or near parturition. In contrast, rats show minimal accumulation in the fetus and the amount of PFOA in the fetal compartment is proportional to maternal dose (Butenhoff et al., 2004a). Furthermore, our cross-foster study shows the presence of PFOA in mice exposed only during the neonatal period and suggests that lactational exposure occurs in mice as occurs in rats (Hinderliter et al., 2005).

Mice are more appropriate for elucidating the pharmacokinetics of both PFOA and precursor compounds than are rats. In female rats, PFOA has a half-life of 2-4 hours (Kudo et al., 2001, Ohmori et al., 2003) and therefore actual fetal and neonatal exposure is thought to be minimal following maternal dosing (Lau et al., 2004, Lau et al., 2006). The significance of the lack of PFOA-induced reproductive toxicity in rats was questioned by Lau et al. (2006) because chronic dosing results in episodic increases in maternal serum and do not appear to reach steady state (Lau et al., 2006). Based on the calculated half-lives in pregnant dams (14.9 days, from the current study), which are similar to published values for male and female mice (Kudo and Kawashima, 2003), our data support that adult mice are absent sex-mediated excretion processes.

Accumulation of PFOA in the developing mouse fetus can result in fetal body burdens that affect post-natal growth and development. We found that following *in utero* exposure, 11 of 36 dams had at least one abnormal pregnancy outcome including still births and/or neural tube

defects. Similarly, Lau et al. (2006) determined that the survival of mouse neonates was highly influenced by *in utero* chronic exposure to PFOA. Since fetal concentrations of PFOA increase temporally after a single oral dose of 8-2 FTOH given to dams, decreased post-natal survival following chronic exposure to PFOA (Lau et al., 2006) may be attributed to the observed increasing concentration during gestation. To our knowledge, this is the first attempt at measuring the maternal-fetal transfer of PFOA following an oral dose of 8-2 FTOH or PFOA.

One potential explanation for the accumulation of PFOA in the fetus after a single exposure is the pH gradient established between the maternal and fetal compartment. Although most cell membranes are generally impermeable to weak, ionized acids (Milne et al., 1958), during pregnancy a pH gradient is established between the maternal and fetal plasma compartments (Nau and Scott, 1986). The more acidic embryonic compartment is able to trap ionized metabolites and therefore may explain the accumulation of PFOA and PFNA noted in this study. Fetal ion-trapping has been demonstrated with 2-methoxyacetic acid (MAA; pka = 3.57) and 5,5'-dimethylozaxolidine-2,4-dione (DMO; pka = 6.13) both weak acids (Terry et al., 1995 and O'Flaherty et al., 1992, respectively). Furthermore, protein binding and possible protein-mediated transport may account for elevated concentrations of these terminal acids in fetal compartments. In a recent attempt to explain varied species half-lives, Andersen and co-workers (2006) demonstrate the feasibility of a reverse renal proximal tubular transport process being responsible for PFOA retention.

In male mice, 8-2 FTOH metabolism into PFOA causes peroxisome proliferation and affects the normal fatty acid metabolism occurring in the liver via peroxisome proliferation and manifests as increased LW:BW ratio (Kudo et al., 2005). However, it should be noted that increases in relative liver weights have also been observed in PPAR-alpha non-responsive

species (Butenhoff et al., 2002) and in PPAR-alpha transgenic knock-out mice (Yang et al., 2002) suggesting an alternative mechanism of liver enlargement.

In our study, 8-2 FTOH and its subsequent metabolism to PFOA cause an increase in the relative liver weight (Tables 5-2 and 5-3) of both dams and neonates. Our maternal data support the temporal effect on relative liver weight as the maternal LW:BW ratio was affected as early as GD13, which is 5 days post-treatment, and continued the duration of the study. Secondly, increases in relative liver weight are induced in the neonates at the earliest time-point investigated suggesting that similar mechanisms of peroxisome proliferation or general liver hypertrophy are occurring following *in utero* exposure to 8-2 FTOH.

Our study shows that, in mice, 8-2 FTOH is a developmental toxicant and toxicity likely results from its rapid metabolism to PFOA and PFNA and/or exposure to intermediate metabolites of 8-2 FTOH. Maternal exposure to 8-2 FTOH can result in birth defects and neonatal relative liver weight gain, suggesting exposure to the developing fetus during the critical period of neural tube closure as well as a mechanism involving induced peroxisome proliferation, respectively. The present study supports the mouse as an appropriate animal model for describing the developmental toxicity of 8-2 FTOH and its metabolites as they relate to humans. Based on rat studies, the developmental toxicity of 8-2 FTOH was thought to be negligible (Mylchreest et al., 2005); however, when research animal models such as mice lack active excretion processes, terminal acid metabolites accumulate in the developing fetus following a single oral exposure. Understanding the metabolism 8-2 FTOH and subsequent distribution of PFOA and PFNA as well as the potential developmental toxicity of intermediate metabolites will greatly advance the science behind the human-health risk assessment of these fluorotelomer based chemicals.

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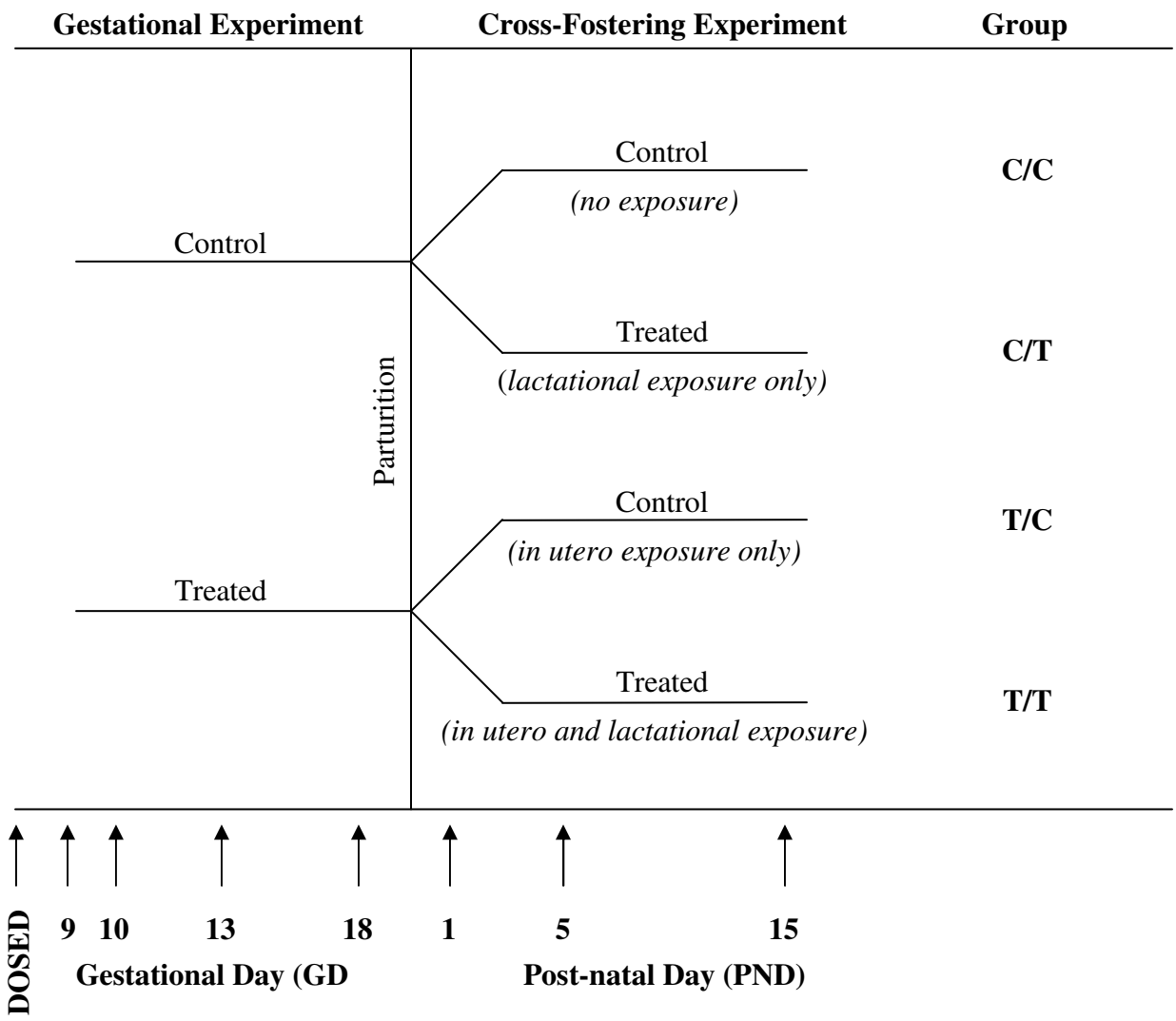

Figure 5-1: Schematic of gestational study and cross-foster study experimental design with resulting exposure groups as well as a time line of data collection points.

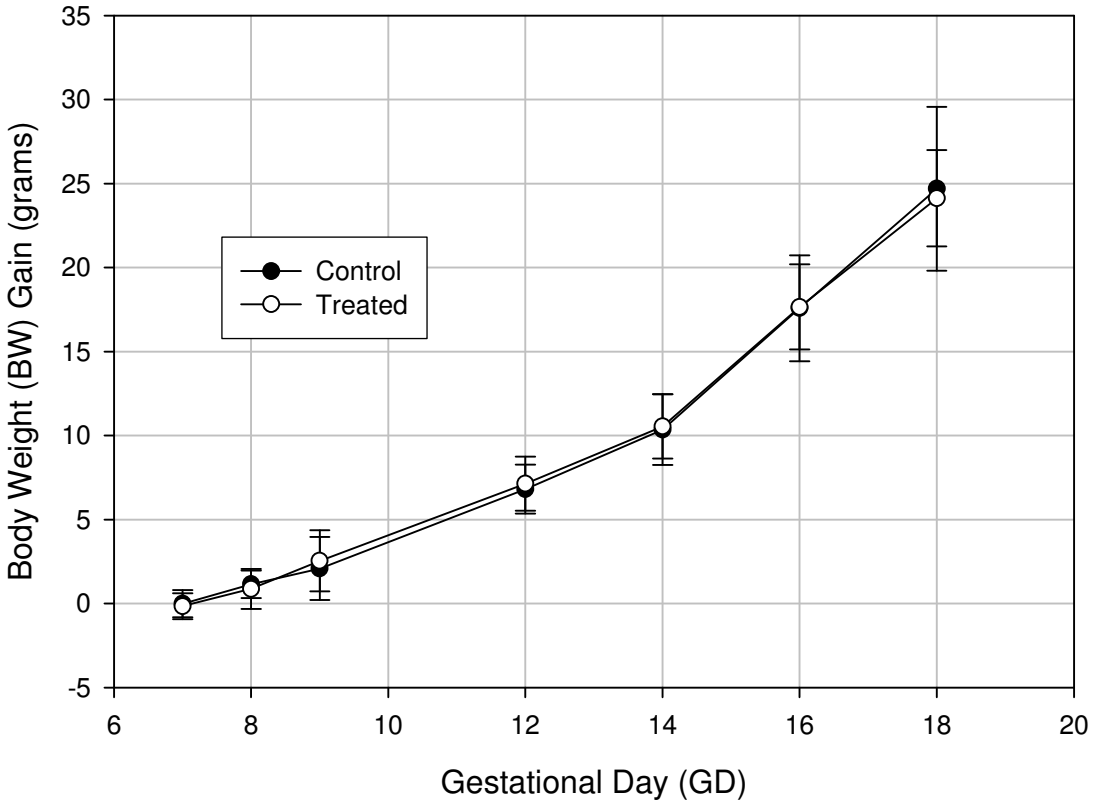

Figure 5-2: Maternal body weight gain throughout gestation after pregnant CD-1 mice received 30 mg/kg BW 8-2 FTOH by gavage on GD8. Data points presented were taken throughout the duration of gestation and represent the mean (n=34 for control and n=36 for treated) while error bars represent the standard deviation (SD). There were no statistical differences ($p < 0.05$) between control and treated dams up to GD18.

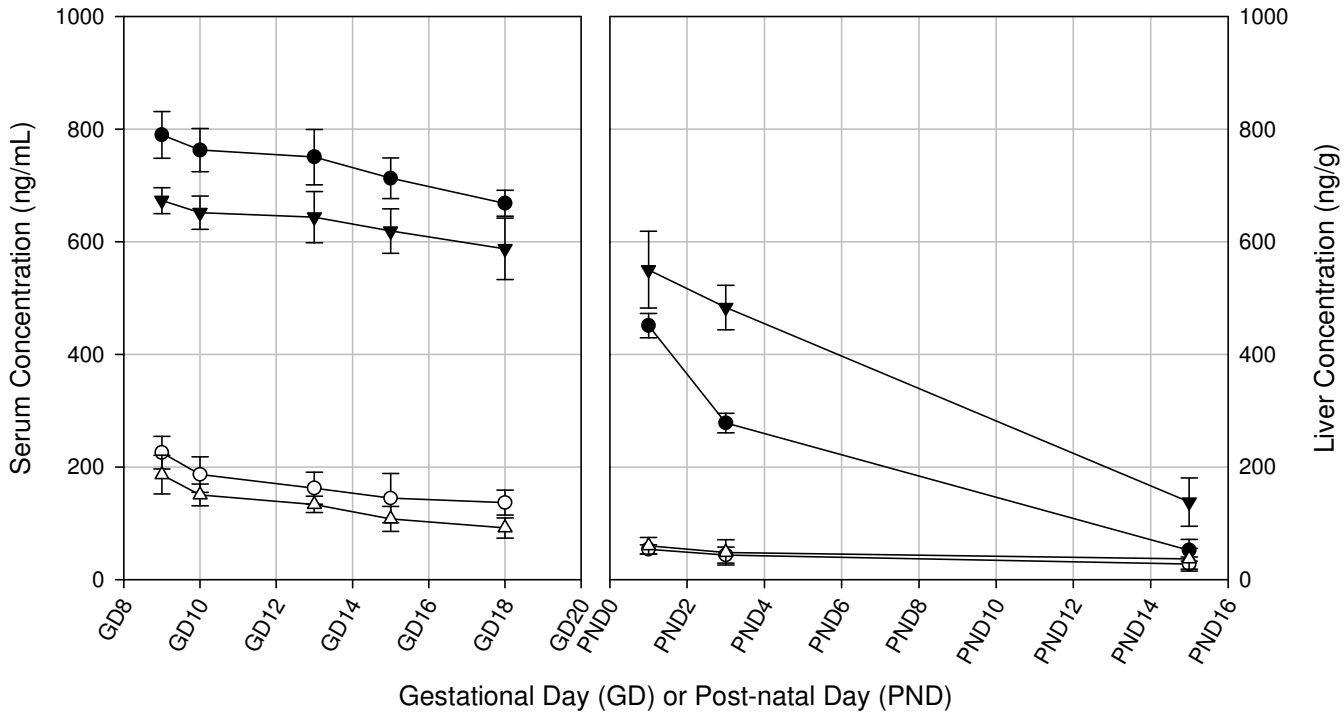

Figure 5-3: Maternal serum (● and ○) and liver (▼and Δ) concentrations of PFOA (solid) and PFNA (open) from CD-1 mice orally exposed to 30 mg/kg body weight on GD8 (n≥5). The graphical break represents parturition in which neonates were sub-divided into cross-fostered treatment groups. Post-natal maternal concentrations are the average from dams treated with 8-2 FTOH irrespective of cross-foster treatment group as no statistical difference in PFOA serum or liver concentrations were detected between control dams (C/C) and those that were given *in utero* treated pups (T/C). Note that 8-2 FTOH was not detected in maternal liver or serum at any time ≥24 hours post-treatment.

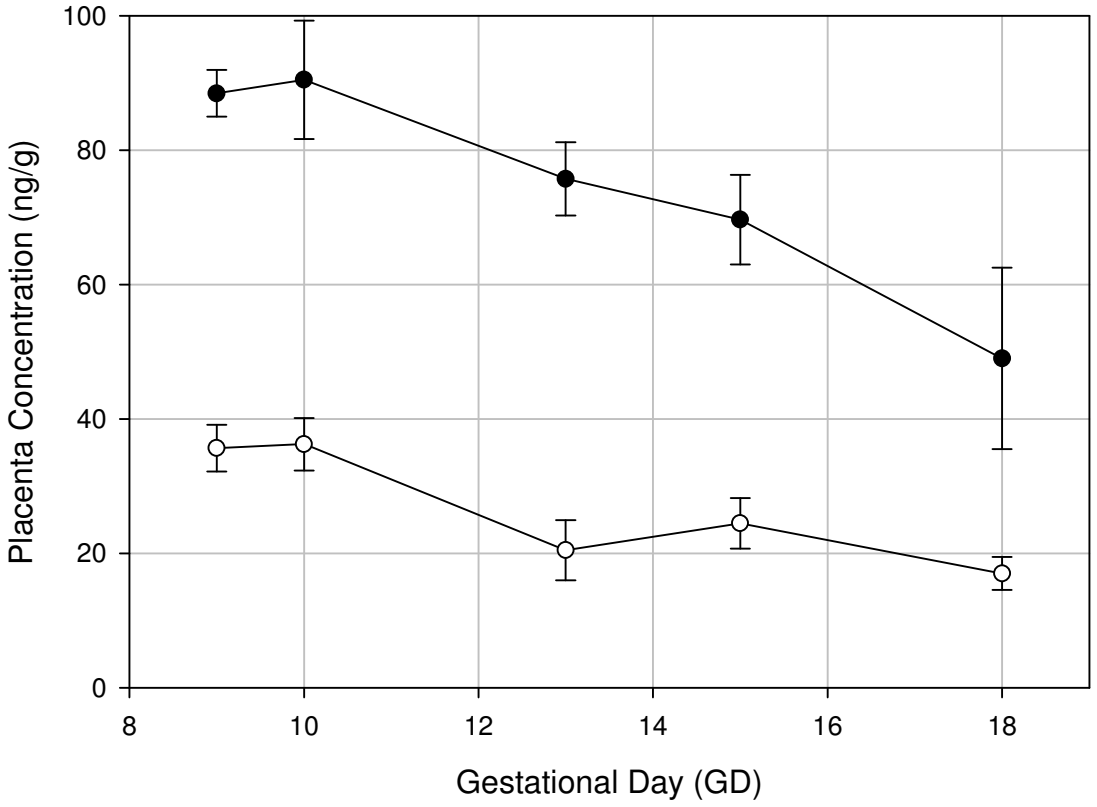

Figure 5-4: Placental Concentrations of PFOA (solid) and PFNA (dotted) from timed-pregnant mice that received a single gavage dose (30 mg 8-2 FTOH/kg body weight) on GD8. Data points represent the average (n≥5) and error bars represent the standard deviation (SD).

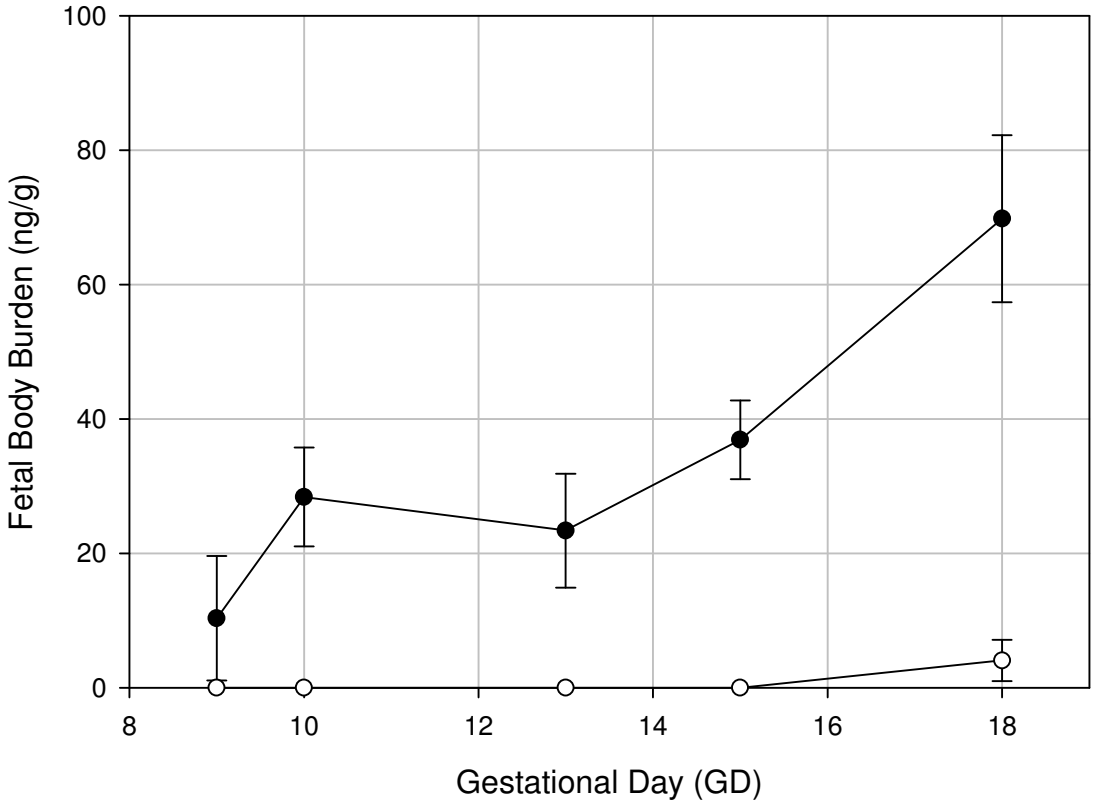

Figure 5-5: 8-2 FTOH metabolites, PFOA (solid line) and PFNA (dashed line), whole-body burden in fetal mice from dams which received 30 mg/kg body weight 8-2 FTOH on GD8. Data points represent the average (n≥5) and error bars represent the standard deviation (SD). Note that PFNA was only above method quantification limits at GD18 and concentrations of PFNA were generally 10 fold less than those determined for PFOA.

Table 5-1: Chemical names, abbreviations, and CAS numbers of PFCs used to determine the metabolism and transport of 8-2 FTOH and its terminal metabolites, PFOA and PFNA in pregnant mice, fetuses and neonates.

Chemical Name	Abbreviation	CAS Number	Structural Formula
8-2 fluorotelomer alcohol	8-2 FTOH	678-39-7	$CF_3(CF_2)_7CH_2CH_2OH$
perfluorooctanoic acid	PFOA	335-67-1	$CF_3(CF_2)_6COOH$
perfluorononanoic acid	PFNA	375-95-1	$CF_3(CF_2)_7COOH$
methyl perfluorooctanoate	PFOA-Me	376-27-2	$CF_3(CF_2)_6COOCH_3$
methyl perfluorononanoate	PFNA-Me	51502-45-5	$CF_3(CF_2)_7COOCH_3$
methyl perfluoroheptanoate	PFHA-Me	14312-89-1	$CF_3(CF_2)_5COOCH_3$
3-(perfluorooctyl) propanol	3-PFOP	1651-41-8	$CF_3(CF_2)_7CH_2CH_2CH_2OH$
8-2 fluorotelomer acid	8-2 FTCA	27854-31-5	$CF_3(CF_2)_7CH2COOH$
8-2 fluorotelomer unsaturated acid	8-2 FTUCA	70887-84-2	$CF_3(CF_2)_6CF=CHCOOH$

Table 5-2: Effects of 8-2 FTOH administration on gestational parameters in CD-1 mice.

Gestational Day	Groups	Maternal Body Weight (g) ± SD[a]	Maternal Liver Weight (g) ± SD	Maternal Liver /Body Weight Ratio (x100)	Fetal Body Weight (g) ± SD
GD 9	Control (n=4)	31.9 ± 0.3	1.59 ± 0.3	4.98	0.06 ± 0.1
	Treated (n=5)	30.8 ± 0.8	1.58 ± 0.4	4.99	0.07 ± 0.1
GD 10	Control (n=4)	32.6 ± 0.4	1.63 ± 0.2	5.00	0.08 ± 0.0
	Treated (n=5)	29.9 ± 0.7[b]	1.47 ± 0.3	4.92	0.09 ± 0.0
GD 13	Control (n=4)	36.5 ± 0.8	1.79 ± 0.2	4.90	0.34 ± 0.0
	Treated (n=5)	34.7 ± 1.9	1.96 ± 0.2	5.65[b]	0.32 ± 0.1
GD 15	Control (n=4)	45.5 ± 1.1	2.05 ± 0.1	4.65	0.82 ± 0.1
	Treated (n=5)	43.9 ± 0.9	2.11 ± 0.3	5.72[b]	0.78 ± 0.0
GD 18	Control (n=4)	55.2 ± 1.3	2.17 ± 0.2	3.93	1.12 ± 0.1
	Treated (n=5)	54.7 ± 1.1	2.67 ± 0.2[b]	4.88[b]	1.05 ± 0.1

[a] SD = standard deviation.
[b] Denotes statistical difference ($p < 0.05$) when compared to controls.

Table 5-3: Effects of *in utero* 8-2 FTOH administration on post-natal parameters in CD-1 mice.

Post-natal Day	Maternal Treatment Group	Maternal Body Weight (g) ± SD[a]	Maternal Liver Weight (g) ± SD	Maternal Liver/Body Weight Ratio (x100)	Neonatal Treatment Group[b]	Neonatal Body Weight (g) ± SD	Neonatal Liver Weight (g) ± SD	Neonatal Liver/Body Weight Ratio (x100)
PND 1	Control (n=10)	31.70 ± 0.7	2.48 ± 0.3	7.82	C/C	1.95 ± 0.1	ND[c]	ND
					T/C	1.83 ± 0.1	ND	ND
	Treated (n=10)	29.30 ± 1.1[d]	2.63 ±0.2	8.97[d]	C/T	1.83 ± 0.2	ND	ND
					T/T	1.86 ± 0.3	ND	ND
PND 3	Control (n=9)	33.2 ± 0.9	2.76 ± 0.2	8.31	C/C	2.53 ± 0.3	0.84 ± 0.1	33.2
					T/C	2.22 ± 0.3	1.04 ± 0.1	46.6[d]
	Treated (n=10)	28.90 ± 1.1[d]	2.77 ± 0.3	9.58[d]	C/T	2.32 ± 0.4	0.91 ± 0.0	39.3
					T/T	2.40 ± 0.3	1.10[d]± 0.1	46.0[d]
PND 15	Control (n=9)	31.6 ± 0.6	3.01 ± 0.3	9.52	C/C	5.18 ± 0.3	1.62 ± 0.1	31.2
					T/C	4.95 ± 0.2	1.74 ± 0.2	35.1[d]
	Treated (n=10)	30.4 ± 1.0	3.39 ± 0.5	11.15[d]	C/T	5.13 ± 0.2	1.62 ± 0.2	31.5
					T/T	4.93 ± 0.2	1.95[d] ± 0.1	39.5[d]

[a] SD = standard deviation.
[b] C/C = no exposure; T/C = *in utero* exposure only; C/T = lactational exposure only; T/T = both *in utero* and lactational exposure.
[c] ND = not determined at this time-point.
[d] Denotes statistical difference ($p < 0.05$) when compared to controls.

Table 5-4: Effects of 8-2 FTOH administration on fetal and neonatal viability in CD-1 mice.

		Litter Size (mean ± SD[a])	% Non-viable (per # fetuses or neonates)	% Non-viable (per # dams)	% with NTDs[b] (per # non-viable)
Gestational Day 15	Control (n=3)	12 ± 3	**0 %** (0/39)[c]	**0 %** (0/3)[d]	**0 %** (0/0)[d]
	Treated (n=5)	12 ± 2	**1.6 %** (1/61)	**20.0 %** (1/5)	**0 %** (0/1)
Gestational Day 18	Control (n=3)	13 ± 2	**0 %** (0/39)[c]	**0 %** (0/3)[d]	**0 %** (0/0)[d]
	Treated (n=6)	12 ± 2	**1.4 %** (1/73)	**16.7 %** (1/6)	**100 %** (1/1)
Post-natal Day 0	Control[d] (n=34)	13 ± 2	**0 %** (0/432)	**0 %** (0/34)	**0 %** (0/0)
	Treated (n=36)	13 ± 2	**3.2 %**[e] (15/469)	**30.6 %**[e] (11/36)	**26.7 %**[e] (4/15)

[a] SD = standard deviation.
[b] NTD = neural tube defect assessed as anencephaly or exencephaly
[c] In the gestational experiment, only gestational days 15 and 18 were examined for gross malformations.
[d] Reproductive indices for CD-1 mice (n=506) are 0.1 % for average number of non-viable fetuses and 0 % NTDs covering June 1999 thru June 2003 (personal communication with Charles River Laboratories, 10/23/06).
[e] Denotes statistical difference ($p < 0.05$) when compared to controls.

Table 5-5: Post-natal body burden and distribution of PFOA and PFNA in neonatal mice following *in utero* exposure to 8-2 FTOH.

		PFOA Concentration (average ± SD[a])			**PFNA Concentration (average ± SD)**		
Post-natal Day	Treatment Group[b]	Whole-body (ng/g)[c]	Serum (ng/mL)	Liver (ng/g)	Whole-body (ng/g)	Serum (ng/mL)	Liver (ng/g)
PND 1	C/C	ND[d]	NA[e]	NA	ND	NA	NA
	C/T	54 ± 10	NA	NA	43 ± 15	NA	NA
	T/C	178 ± 18	NA	NA	88 ± 9	NA	NA
	T/T	223 ± 19	NA	NA	91 ± 16	NA	NA
PND 3	C/C	ND	ND	ND	ND	ND	ND
	C/T	25 ± 15	57 ± 11	48 ± 9	42 ± 9	31 ± 5	28 ± 6
	T/C	142 ± 7	207 ± 29	125 ± 16	33 ± 11	47 ± 1	22 ± 5
	T/T	156 ± 20	208 ± 16	131 ± 27	56 ± 11	38 ± 2	42 ± 8
PND 15	C/C	NA	ND	ND	NA	ND	ND
	C/T	NA	58 ± 3	49 ± 11	NA	33 ± 5	12 ± 4
	T/C	NA	96 ± 6	79 ± 17	NA	45 ± 8	26 ± 8
	T/T	NA	102 ± 28	89 ± 12	NA	37 ± 7	32 ± 3

[a] SD = standard deviation.
[b] Pups were cross-fostered with dams that had been treated during gestation with 8-2 FTOH (T) or vehicle (C) resulting in four treatment groups in which the first letter represents *in utero* (fetal) exposure and the second represents lactational (neonatal) exposure: C/C, T/C, C/T, T/T.
[c] Whole neonatal homogenates were processed due to amount of tissue needed for extraction and perfluorinated chemical analysis.
[d] ND = not detected. Amount determined was below method quantification limits.
[e] NA = not applicable. Due to small size of the neonate only whole body concentrations were determined for PND1. Liver and serum concentrations were determined at PND3 and PND15.

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CHAPTER 6

SUMMARY

The overall goal of this research was to investigate the contribution of 8-2 fluorotelomer alcohol (FTOH) metabolism to the presence of perfluorooctanoic acid (PFOA) detected in mammals. Because exposure to precursor compounds is highly plausible and not well understood, 8-2 FTOH metabolism was compared in adult male, female and pregnant CD-1 mice. Understanding 8-2 FTOH metabolism and distribution in the mammalian system will further the knowledge of human-health risks associated with exposure to 8-2 FTOH and other precursor compounds. Secondly, investigating the developmental toxicity of precursor perfluorinated chemicals and their metabolites will add valuable information to help refine the risk assessment of 8-2 FTOH and PFOA. Ultimately, the knowledge gained herein will aid in model development as well as predicting the effects of exposure to 8-2 FTOH at environmentally relevant concentrations and its subsequent metabolism to PFOA.

Method Development

Because simultaneous quantification was needed for volatile and non-volatile perfluorinated chemicals (PFCs) in complex matrices, a GC/MS method was developed and tested based on selected ion monitoring of perfluorinated alkyl parent chain fragment ions (Objective 1, Chapter 3). Although the method requires a derivatization step, combined GC/MS analysis of PFCAs and FTOHs increases analytical efficiency and decreases sample analysis

time. We describe a method for the simultaneous determination of several FTOHs and PFCAs by a GC/MS analysis in selected ion monitoring (SIM) mode. A selective and sensitive method for analysis of perfluorinated chemicals (PFCs) in mammalian tissue matrices included monitoring for mass fragments 131, 169, 231, and 331 *m/z*, which are all common to the perfluorinated alkyl chains of this class of compounds. Although average instrumental detection limits and method quantification limits are higher than currently reported methods, this method has the advantage of simultaneously detecting both volatile and non-volatile fluorotelomer-based chemicals in complex matrices.

Metabolism and Distribution of 8-2 FTOH in Male and Female CD-1 Mice

The second objective of our study was to investigate the metabolism and distribution of 8-2 FTOH in male CD-1 mice (Chapter 4). Mice were chosen for this study, instead of rats, because it has been hypothesized that rats possess an anion-transport mediated excretion process not seen in humans. CD-1 mice do not use this excretory process and are, consequently, a more suitable research model for humans when studying the metabolism and distribution of the specified compounds.

CD-1 mice received a gavage dose (10-30 mg 8-2 FTOH/kg BW) in a propylene glycol/water (1:1) vehicle on day 0. 8-2 FTOH was rapidly metabolized and no longer present by 24 hrs post-treatment and the estimated half-life ($t_{1/2}$) of 8-2 FTOH in serum of male mice was 6.2 hrs. In male mice, maximal PFOA concentrations in serum and liver were at 12 hrs post-treatment and temporally decreased up to 120 hrs. The maximum concentrations in serum and liver of female mice also occurred by 24 hrs post-treatment. Using linear regression, the

calculated $t_{1/2}$s in male mice and female mice were comparable, suggesting a lack of sex-mediated excretion processes.

Metabolism and Distribution of 8-2 FTOH in Pregnant CD-1 Mice

Because PFOA has been associated with developmental effects in mice, our third objective was to investigate the metabolism, distribution, and developmental outcomes in pregnant CD-1 mice (Chapter 5). 8-2 FTOH is rapidly metabolized in pregnant mice following a single oral exposure to 30 mg 8-2 FTOH/kg BW. The maximum concentrations in serum and liver of pregnant mice occurred by 24 hrs post-treatment. In contrast to non-pregnant female and male mice, during gestation (GD 8-18), the $t_{1/2}$ was longer, but values after parturition were similar to non-pregnant females. Pregnant mice retain PFOA longer in serum than non-pregnant females suggesting that physiological changes during pregnancy increase PFOA retention and may implicate fetal toxicity.

Maternal-Fetal and Neonatal Transfer of PFOA and PFNA

Finally, following *in utero* exposure to 8-2 FTOH on GD8, PFOA was transferred to the developing fetuses as early as 24 hours post-treatment while PFNA was quantifiable only at GD18. Post-partum, maternal serum PFOA concentrations decreased from post-natal day (PND) 1 to PND15 and PFNA concentrations, although 5-fold less, exhibited a similar trend. Immediately after birth, pups were cross-fostered with dams that had been treated during gestation with 8-2 FTOH (T) or vehicle (C). On PND1 thru PND2, neonatal whole-body homogenate concentrations of PFOA from T/T and T/C were comparable as were concentrations in neonatal liver and serum from PND3 to PND15. Based on detectible amounts of PFOA in

neonatal serum in the C/T group on PND3 and on PND15, we suggest the neonates were exposed through lactation. In conclusion, exposure of neonates to PFOA and PFNA occur both pre- and post-natally following maternal 8-2 FTOH exposure on GD8.

Future Goals of Research

Understanding the metabolism 8-2 FTOH and subsequent distribution of PFOA and PFNA as well as the potential developmental toxicity of intermediate metabolites will greatly advance the science behind the human-health risk assessment of these fluorotelomer based chemicals. Throughout the course this study, various areas requiring further investigation were uncovered. Because there appears to be both similarities and differences in species metabolism of 8-2 FTOH, *in vitro* studies could be conducted to complement the data obtained from *in vivo* studies. Secondly, mechanistic studies can be employed to elucidate the mechanisms of both 8-2 FTOH metabolism and its induction of neural tube defects. Furthermore, genomic and metabolomic studies would greatly enhance the knowledge of 8-2 FTOH metabolism.

CHAPTER 7

CONCLUSIONS

We conclude, 1) monitoring perfluoroalkyl chain fragments allows the simultaneous detection and quantification of perfluorinated chemicals. Based on the proposed metabolism of 8-2 FTOH shown in Figure 3, Chapter 2, 2) β-oxidation is the major route of 8-2 FTOH metabolism in mice; however, α-oxidation is also occurring, 3) both male and female mice achieve maximal concentrations of PFOA by 24 hours after a single oral exposure of 8-2 FTOH and, 4) similar $t_{1/2}$s in male and female CD-1 mice suggests that, unlike rats, there is little or no sex-mediated differences in excretion processes for PFOA. Finally, 5) 8-2 FTOH metabolism is similar in pregnant CD-1 mice; however, they retain PFOA longer than male or female mice, 6) both fetal (gestational) and neonatal (lactational) exposure results from maternal transfer of 8-2 FTOH or its metabolites, and, 7) *in utero* exposure to 8-2 FTOH results in increased mortality as well as increased incidence of neural tube defects in neonates.