Study Title

Ammonium Perfluorooctanoate:
Age Effect on the PFOA Plasma Concentration in Post-Weaning Rats Following Oral Gavage

TEST GUIDELINES: None applicable

AUTHOR: Paul M. Hinderliter, Ph.D.

STUDY COMPLETED ON: December 2, 2004

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LABORATORY PROJECT ID: DuPont-15302

WORK REQUEST NUMBER: 15339

SERVICE CODE NUMBER: 1389

SPONSOR: E.I. du Pont de Nemours and Company
Wilmington, Delaware 19898
U.S.A.

and

3M Company
3M Center Building
St. Paul, MN 55144-1000

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, except for the item documented below. The item listed did not impact the validity of the study.

- No conduct audit was conducted on this study, however, all activities were performed by experienced, trained personnel. All work was documented in the study records.

Study Director: _____ 02-Dec-2004
Paul M. Hinderliter, Ph.D. Date
Research Toxicologist

QUALITY ASSURANCE DOCUMENTATION

Work Request Number: 15339
Study Code Number: 1389

The conduct of this study has been subjected to periodic Quality Assurance inspections. The dates of inspection are indicated below.

Phase Audited	*Audit Dates*	*Date Reported to Study Director*	*Date Reported to Management*
Protocol:	July 22, 23, 2004	July 23, 2004	July 23. 2004
Report/Records:	October 28, 29, 2004	October 29. 2004	November 24, 2004

Reported by: Joseph C Hamill 02-Dec-2004
Joseph C. Hamill
Quality Assurance Auditor
Date

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Analytical Evaluation by: Janet C. Maslanka — 30-Nov-2004
Janet C. Maslanka, B.S.
Analytical Chemist
Date

Approved by: 30-Nov-2004
Gary W. Jepson, Ph.D.
Research Manager
Date

Issued by Study Director: 02-Dec-2004
Paul M. Hinderliter, Ph.D.
Research Toxicologist
Date

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STUDY INFORMATION

9th Collective Nomenclature: Octanoic acid, pentadecafluoro-, ammonium salt

Synonyms/Codes:
- Ammonium perfluorooctanoate
- FC-143 FLUORAD Brand Fluorochemical Surfactant (3M Company, Specialty Materials)
- C-8
- APFO
- Perfluorooctanoate, ammonium salt
- PFOA
- H-24921
- Lot 332 (3M Specialty Materials) (Lot No.)

Haskell Number: 24921

CAS Registry Number: 3825-26-1

Purity: 95.2% – 97.99%
Straight chain: 77.6%
Branched: 12.6% internal monomethyl (non-alpha)
9% isopropyl
0.2 % tert-butyl
0.1% gem-dimethyl
0.1% alpha monomethyl

Known Impurities: C_4 ($C_3F_7CO_2^- NH_4^+$), 0.01%
C_5 ($C_4F_9CO_2^- NH_4^+$), 0.03%
C_6 ($C_5F_{11}CO_2^- NH_4^+$), 0.43%
C_7 ($C_6F_{13}CO_2^- NH_4^+$), 0.57%
C_9 ($C_8F_{17}CO_2^- NH_4^+$), 0.16%
Monohydro APFO, 0.09%
Monounsaturated APFO, 0.72%
Undefined (possibly) substituted perfluorocyclo species, 0.2% cyclopentyl, and 0.1% cyclohexyl

Physical Characteristics: White solid

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Study Initiated/Completed: July 8, 2004 / (see report cover page)

Experimental Start/Completion: August 9, 2004 / October 13 2004

SUMMARY

Perfluorooctanoate (PFOA) plasma concentrations were measured in immature (3, 4 or 5 weeks of age) rats following single oral gavage doses at 10 and 30 mg/kg. Plasma concentrations varied significantly between experiments, especially when compared to a prior study with 4-week old animals. In the current study, male rat PFOA plasma concentrations are relatively constant in male rats across age groups, varying only with dose level. Female rat plasma concentrations decreased with increasing age, but was only statistically significantly at the 30 mg/kg dose level. PFOA concentrations in urine, while not normalized for total elimination, indicated more rapid urinary excretion in female rats at all ages. The plasma concentrations from the supplemental group, however, are significantly different from the main study levels for both male and female rats. The plasma concentration variability in 4-week old rats suggests an unknown physiological variability in animals younger than 5 weeks. As a result, the relationship between age and PFOA plasma concentration is undefined in 3-4 week-old rats following oral dosing with 10 or 30 mg/kg PFOA.

A. Plasma

PFOA plasma concentrations in male rats were not significantly different by sample time (at 2 and 24 hours) or by animal age (3, 4 or 5 weeks) at either 10 or 30 mg/kg, except at 2 hours and 5 weeks of age. The PFOA plasma concentrations in female rats were significantly lower at 24 hours post dosing (compared to 2 hours) at all ages and doses tested. Female plasma concentrations were also lower for 5 week-old rats than 3 and 4 week-old rats at both sample times and doses ($p < 0.01$). The PFOA plasma concentrations following a 30 mg/kg dose were approximately 1.5 to 4 times higher than those observed following a 10 mg/kg dose.

In a prior study, male and female rat PFOA plasma concentrations were measured at 24 hours post-dosing following a single 10 mg/kg oral gavage dose. PFOA plasma concentrations are consistent with the current study for both sexes at 5 weeks of age but not at 4 weeks of age (3-week old animals were not previously measured). The standard deviations are small in both studies, indicating that both are internally consistent. The only known variation between the two studies is whether the rats were fasted before dosing. The supplemental rats were added to examine the effect of fasting on the PFOA plasma concentrations in 4-week old rats. Fasting increased the PFOA plasma concentrations as expected but the nominal vales are different from either the current study or the prior study. Given the consistency in the 5-week old rat PFOA plasma concentrations, it appears that there is an unknown physiological variability in rats younger than 5 weeks which impacts the pharmacokinetics of PFOA.

B. Urine

There were significant differences in PFOA urine concentrations with age, dose, and sex. Female rats showed higher PFOA urine concentrations, and these increased with age. Male rats showed the opposite, with urine concentrations decreasing with age. Both sexes showed higher

(2.5-6.5 times) urine concentrations at 30 mg/kg compared to 10 mg/kg doses. No attempt was made to quantify urinary output (volume), and, because creatinine levels were not measured, adjustments for urine concentration could not be performed.

INTRODUCTION

Ammonium perfluorooctanoate (APFO) is a synthetic perfluorinated octanoic acid used as an industrial surfactant. Elimination of the disassociated anion, perfluorooctanoate (PFOA) in rats is sex-dependent and appears to be under hormonal regulation. PFOA elimination is much faster in female rats than in male rats, down-regulated by testosterone in both female and castrated male rats, and up-regulated by estradiol in the male rats.[1-4] A prior study conducted at DuPont Haskell Laboratory found sex-dependent PFOA elimination in 4-8 week old rats.[5] The sex difference was least at 4 weeks of age, at time period just prior to sexual maturation in the rat. The objective of the this study was to determine if 3-week old male and female rats eliminate PFOA from blood at a rate different than that observed in 4- or 5-week old rats. Male and female post-weaning rats at different ages were dosed with APFO, and the PFOA plasma concentrations were compared at 2 and 24 hours post-dosing. At 24 hours after dosing, the sex difference in PFOA elimination in sexually mature rats was easily distinguished via the plasma concentration of PFOA.[6] Urine was collected for 24 hours and analyzed for PFOA concentration. This study was designed to evaluate limited kinetic endpoints exclusive of determination of elimination rate constants.

MATERIALS AND METHODS

A. Test Substance

APFO was obtained from 3M (St. Paul, MN) and assigned Haskell Laboratory Number H-24921 upon receipt. Available information on the purity, composition, contaminants, synonyms, hazards, and hazardous material classification(s) were provided by the vendor and documented in the study records and/or report.

B. Test System

Male and female Crl:CD®(SD)IGSBR rats were obtained from Charles River Laboratories, Inc., Raleigh, North Carolina. Animals for groups I-IV arrived as litters with dams. The Sprague-Dawley rat was chosen for this study because of the extensive experience with this strain and its suitability with respect to longevity, sensitivity, and low incidence of spontaneous diseases. Furthermore, the Sprague-Dawley rat has been used previously for toxicokinetic testing of PFOA and other fluorinated test materials.[2,5-8]

At the time of dosing, rats were approximately 3, 4, and 5 weeks of age and the weight variation did not exceed ±20% of the mean weight by dose group and sex. Information on the cage labels included the Haskell animal number and an individual identification number assigned to each rat. The individual identification number was placed on the tail of each rat by tattoo or tail mark.

C. Animal Husbandry

1. Housing

Upon arrival at Haskell Laboratory, rats were removed from shipping cartons and housed in appropriate cages according to Standard Operating Procedures unless specified in the study records. Rats were maintained under quarantine for at least three days unless approved otherwise by the site veterinarian, had at least one recorded weight gain, and no abnormalities detected. After the quarantine period, rats were selected for study.

During pretest, dams were housed with their litters in polycarbonate pens containing bedding. Throughout the dosing period with test substance, the rats were housed individually in appropriate cages according to the SOP. During the urine collection period, rats were housed in stainless steel wire mesh cages.

2. Cage and Cage Rack Change

Due to the length of the study, no cage and cage rack changes were required.

3. Environmental Conditions

Animal rooms were maintained at a temperature of 18-26°C (targeted to 22-24°C) and a relative humidity of 30-70% (targeted to 40-60%). Animal rooms were artificially illuminated (fluorescent light) on an approximate 12-hour light/dark cycle. Unless judged by the study director or the laboratory veterinarian to have significantly affected the results of the study, the relative humidity and temperature ranges in the housing rooms were recorded but were not included in the final report.

4. Feed and Water

All rats were provided tap water *ad libitum* and fed PMI® Nutrition International, LLC Certified Rodent LabDiet® 5002 *ad libitum*. Rats were not fasted prior to dosing due to their age.

5. Animal Health and Environmental Monitoring Program

As specified in the Haskell Laboratory animal health and environmental monitoring program, the following procedures were performed periodically to ensure that contaminant levels were below those that would be expected to impact the scientific integrity of the study:

- Water samples were analyzed for total bacterial counts, and the presence of coliforms, lead, and other contaminants.
- Samples from freshly washed cages and cage racks were analyzed to ensure adequate sanitation by the cagewashers.

Certified animal feed was used, guaranteed by the manufacturer to meet specified nutritional requirements and not to exceed stated maximum concentrations of key contaminants, including

specified heavy metals, aflatoxin, chlorinated hydrocarbons, and organophosphates. The presence of these contaminants below the maximum concentration stated by the manufacturer were not expected to impact the integrity of the study.

The animal health and environmental monitoring program was administered by the attending laboratory animal veterinarian. Evaluation of these data did not indicate any conditions that affected the validity of the study.

D. Pretest Period

Upon arrival at Haskell Laboratory, all rats were housed in quarantine. The rats were:

- quarantined for at least 3 days.
- identified temporarily by cage identification.
- weighed at least 3 times during quarantine and once prior to initiation of exposures.
- observed with respect to weight gain and any gross signs of disease or injury.

The rats were released from quarantine by the laboratory animal veterinarian or designee based on body weights and clinical signs.

Rats that were accidentally killed or removed from study during the pretest period were discarded without necropsy. Rats that were found dead or were sacrificed *in extremis* during the pretest period were given a gross examination to check for the presence of disease. The results were not included in the final report unless considered significant to the evaluation of the study.

E. Assignment to Groups

Rats were selected for use on study based on adequate body weight gain and freedom from any clinical signs of disease or injury. They were distributed by computerized, stratified randomization into study groups as designated in the Study Design, so that there were no statistically significant differences among group body weight means. Rats for groups I-IV were also randomized by dam to ensure even distribution. The weight variation of selected rats did not exceed ± 20% of the mean weight. The first 5 rats in each group were designated for sacrifice at the 2 hours and the remaining rats in each group were designated for sacrifice at 24 hours.

Each rat was assigned a unique 6-digit Haskell animal number and an individual cage identification number. The last 3 digits of the Haskell animal number was tattooed on the tail of each rat. The Haskell animal number and cage identification number were both included on the cage label.

Rats that were not assigned to a test group, including the dams from groups I-IV, were released for other laboratory purposes or sacrificed by carbon dioxide asphyxiation and discarded without anatomic pathology evaluation, at the discretion of the study director.

F. Main Study

1. Animals

The experiments were composed of the following animal groups:

Group		Dose	Age[a]		Number of Rats[b]	
Male	Female	(mg/kg)[c]	Weeks	Days	Male	Female
I	II	10	3	21	10	10
III	IV	30	3	21	10	10
V	VI	10	4	28	10	10
VII	VIII	30	4	28	10	10
IX	X	10	5	35	10	10
XI	XII	30	5	35	10	10

[a] Animals were ±1 day from the target age at the time of dosing. The actual age at dosing was documented in the study records and/or final report.
[b] 5/sex/group sacrificed at 2 and 24 hours post-dose for blood collection.
[c] Given as a single gavage dose

2. Dose Preparation, Analysis, and Rates

The test substance was administered as a single oral gavage dose. This route was chosen because it is the route most commonly used for toxicity studies of APFO. APFO was mixed with NANO-pure water to achieve the target dose concentrations. Dose levels of 10 and 30 mg APFO/kg body weight were used with a dose volume of approximately 4 mL/kg body weight.

Dose was prepared on the day of use or prior to the day of use and stored under appropriate conditions.

HPLC-MS methods were used to confirm PFOA concentration in the dose solutions.

3. Sacrifice and Blood Sampling

Rats were sacrificed by CO_2 asphyxiation and blood collected from the vena cava or by cardiac puncture.

4. Experimental Procedure

Rats were obtained from the vendor and administered PFOA at dose levels described above. Two hours after dosing, 5 rats/sex/group were sacrificed and whole blood collected. Whole blood samples were placed into EDTA tubes and maintained on wet ice. Plasma was separated by centrifugation and frozen at <-10°C until analysis.

The remaining 5 rats/sex/group were placed in metabolism cages for urine collection. Urine was collected for 24 hours after dosing. At 24 hours, all remaining rats were sacrificed and whole blood collected. Blood was handled and separated as above.

No rats were found dead or were sacrificed *in extremis*.

G. Supplemental Study

Four additional male and four additional female rats were added to groups V and VI. Two of the male and two of the female rats were fasted overnight for approximately 12 hours before dosing with test substance. Dose preparation and administration was the same as in the main study. All rats were sacrificed at 24 hours post dosing and whole blood collected. Blood was handled and separated as above. HPLC-MS method was used to determine plasma concentrations of PFOA.

H. Analytical Methods

1. Verification of Dose Solution

Methods for verification of dose solution are detailed in Appendix A.

2. Extraction of PFOA from Plasma for LC/MS Analysis

Plasma samples were processed by protein precipitation (PPT) using Isolute Array protein precipitation columns (Jones Chromatography, Lakewood, CO). A 0.5 µg/mL solution of perfluorononanoic acid (Aldrich Chemicals, Milwaukee, WI) in acetonitrile (ACN) was used as an internal standard for quantitation of PFOA. Plasma samples were thawed, and a 20 µL aliquot of each sample was applied to the PPT array. The plasma samples were precipitated by adding appropriate dilution rate volumes of ACN/internal standard solution to the PPT array. Dilution rates, ranging from 1:10 (180 µL of internal standard solution) to 1:200 (3980 µL of internal standard solution), were utilized in order to capture the sample concentrations within the standard curve parameters. The array was slowly eluted under vacuum into a 96-well receiver plate, centrifuged at ~3000 rpm for 5 minutes, and extracts were analyzed by LC/MS.

3. Instrumentation

System:	Waters 2795 Liquid Chromatograph, equipped with quaternary pump, column heater, and autosampler	
Detector:	Quattro Micro Mass Spectrometer	
Mode:	MRM	
Source:	Negative Electrospray	
LM 1 resolution:	10.0	
HM 1 resolution	10.0	
Ion energy 1:	1.0	
Entrance:	5	
Collision:	2	
Exit:	5	
LM 2 resolution:	14	
HM 2 resolution:	14	
Ion energy 2:	2.0	
Multiplier (V):	650	
Capillary (kV):	2.0	
Cone (V):	15	
Extractor (V):	1.0	
RF lens (V):	0	
Source temperature:	130°C	
Desolvation temperature:	350°C	
MS Method:		
Mode:	MRM, 2 transitions	
Time:	0-10.0 min	
Ch1:	413.00 → 369.00	
	Dwell:	0.25 sec
	Collision energy:	15 eV
Ch2:	463.00 → 419.00	
	Dwell:	0.25 sec
	Collision energy:	15 eV

4. Chromatographic Methods

Method:	Plasma concentration analysis
Column:	Waters Xterra MS C18, 2.1x30mm, 2.5 µm
Column temperature:	Ambient
Mobile phases:	A: 50 mM Ammonium Acetate B: Acetonitrile

Gradient:

Time (min)	%B
0.0	5
0.5	10
10.0	100
10.9	100
11.0	10

Flow rate:	0.25 mL/min
Stop time:	11.0 min
Injection volume:	5 µL

I. Statistical Analyses

Group data is represented as Mean ± SD. Where appropriate, statistical significance was assessed by the Student's t-test.

RESULTS AND DISCUSSION

A. Dose Solution Analysis

(Appendix A)

The concentrations of the test substance in the dose solutions were verified by LC/MS method as described in Materials and Methods, Section F. Standards for dose verification and the dose solutions were prepared by different personnel. The determined mean PFOA concentrations of the dose solutions were all within 20% of the targeted concentration (2.5 and 7.5 mg/mL, Appendix A). PFOA was stable in the vehicle for at least 5 hours.

B. Plasma Concentration of PFOA

(Tables 1-2, Figure 1, Appendices B-D)

1. Male Rats

The PFOA plasma concentrations were not significantly different by sample time (at 2 and 24 hours) or by animal age (3, 4 or 5 weeks) at either 10 or 30 mg/kg, except at 2 hours and 5 weeks of age ($p<0.01$ based on the results of unpaired t-test at 99% confidence interval). In some groups the 24-hour post dosing concentrations were higher, but this was not unexpected due to the long plasma elimination of PFOA in male rats.[6] Individual PFOA plasma concentrations ranged from 21.00 to 48.85 µg/mL for the 10 mg/kg dose and from 49.51 to 153.89 µg/mL for the 30 mg/kg dose. PFOA plasma concentrations following a 30 mg/kg dose were approximately 2-3 times higher than those observed following a 10 mg/kg dose.

2. Female Rats

PFOA plasma concentrations were significantly lower at 24 hours post dosing (compared to 2 hours) in all ages and doses tested. PFOA plasma concentrations were also lower at 5 weeks than at 3 or 4 weeks at both sample times and doses ($p < 0.01$). PFOA plasma concentrations following a 30 mg/kg dose were approximately 1.5 to 4 times higher than those observed following a 10 mg/kg dose.

3. Supplemental Rats

Individual PFOA plasma concentrations in the male rats were 64.95 and 30.00 µg/mL for the fasted and non-fasted animals, respectively. PFOA plasma concentrations in the female rats were 68.16 and 26.54 µg/mL for the fasted and non-fasted animals, respectively.

In a prior study,[5] male and female rat PFOA plasma concentrations were measured at 24 hours post-dosing following a single 10 mg/kg oral gavage dose. PFOA plasma concentrations are consistent with the current study for both sexes at 5 weeks of age but not at 4 weeks of age (3-week old animals were not previously measured). The standard deviations are small in both studies, indicating that both are internally consistent. The only known variation between the two studies is whether the rats were fasted before dosing. The supplemental rats were added to examine the effect of fasting on the PFOA plasma concentrations in 4-week old rats. Fasting increased the PFOA plasma concentrations as expected but the nominal vales are different from either the current study or the prior study. Given the consistency in the 5-week old rat PFOA plasma concentrations, it appears that there is an unknown physiological variability in rats younger than 5 weeks which impacts the pharmacokinetics of PFOA.

C. Urine Concentration of PFOA

(Tables 1-2, Figure 2, Appendices E-F)

PFOA urine concentrations differed significantly with age, dose and sex ($p < 0.01$). Female rats had higher PFOA urine concentrations than males and the female PFOA urine concentrations increased with age. Male rats showed the opposite with urine concentrations decreasing with age. Both sexes had higher (2.5 to 6.5 times) PFOA urine concentrations at 30 mg/kg compared to 10 mg/kg doses. No attempt was made to quantify urinary output (volume) or standardize for creatinine levels.

CONCLUSIONS

Perfluorooctanoate (PFOA) plasma concentrations were measured in immature (3, 4 or 5 weeks of age) rats following single oral gavage doses at 10 and 30 mg/kg. Plasma concentrations varied significantly between experiments, especially when compared to a prior study with 4-week old animals. In the current study, male rat PFOA plasma concentrations are relatively constant in male rats across age groups, varying only with dose level. Female rat plasma concentrations decreased with increasing age, but was only statistically significantly at the 30 mg/kg dose level. PFOA concentrations in urine, while not normalized for total elimination, indicated more rapid urinary excretion in female rats at all ages. The plasma concentrations from the supplemental group, however, are significantly different from the main study levels for both male and female rats. The plasma concentration variability in 4-week old rats suggests an unknown physiological variability in animals younger than 5 weeks. As a result, the relationship between age and PFOA plasma concentration is undefined in 3-4 week-old rats following oral dosing with 10 or 30 mg/kg PFOA.

RECORDS AND SAMPLE STORAGE

A sample of the test substance will be collected for archive purposes and retained at Haskell Laboratory, Newark, Delaware. Specimens (if applicable), raw data, and the final report will be retained at Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware.

REFERENCES

1. Ohmori K., Kudo, H., Katayama, K., and Kawashima, Y. (2003). Comparison of the toxicokinetics between perfluorocarboxylic acids with different carbon chain length. Toxicology 184, 135-140.

2. Vanden Heuvel, J.P., Davis, J.W., Sommers, R., and Peterson, R.E. (1992) Renal excretion of perfluorooctanoic acid in male rats: inhibitory effect of testosterone. J. Biochem. Toxicol. 7, 31-36.

3. Kudo, N., Suzuki, E., Katakura, M., Ohmori, K., Noshiro, R., and Kawashima, Y. (2001) Comparison of the elimination between perfluorinated fatty acids with different carbon chain length in rats. Chem. Biol. Interact. 134, 203-216.

4. Ylinen, M., Hanhijarvi, H., Jaakonaho, J., and Peura, P. (1989) Stimulation by oestradiol of the urinary excretion of perfluorooctanoic acid in the male rat. Pharmacol. Toxicol. 65, 274-277.

5. DuPont Haskell Laboratory (2003). Ammonium Perfluorooctanoate: Age Effect on the Plasma Concentration in Post-Weaning Rats Following Oral Gavage. Unpublished report, DuPont-13267.

6. DuPont Haskell Laboratory (2003). Perfluorooctanoic Acid: Toxicokinetics in the Rat. Unpublished report, DuPont-7473.

7. DuPont Haskell Laboratory.(1997). Hazard Characterization for Human Health C8 Exposure. Unpublished report.

8. Vanden Heuvel, J.P., Kuslikis, B.I., Van Rafelghem, M.J., and Peterson, R.E. (1991). Tissue distribution, metabolism, and elimination of perfluorooctanoic acid in male and female rats. J. Biochem. Toxicol. 6, 83-92.

TABLES

TABLES

EXPLANATORY NOTES

ABBREVIATIONS:

SD	standard deviation

Table 1: Plasma and urine PFOA concentrations in male rats dosed via oral gavage with 10 or 30 mg/kg PFOA

Data expressed as µg/mL

			Plasma				Urine	
	Age	Dose	2 Hours Post-Dose		24 Hours Post-Dose		24 Hours Post-Dose	
Group	(weeks)	(mg/kg)	Mean	SD	Mean	SD	Mean	SD
I	3	10	41.87	4.01	34.22	7.89	9.57	4.86
V	4	10	39.92	4.45	42.94	5.33	4.53	2.45
IX	5	10	26.32	6.89	40.60	3.69	4.03	2.36
III	3	30	120.65	12.78	74.16	18.23	51.76	28.86
VII	4	30	117.40	18.10	100.81	13.18	28.70	18.84
XI	5	30	65.66	15.53	113.86	23.36	15.65	6.24

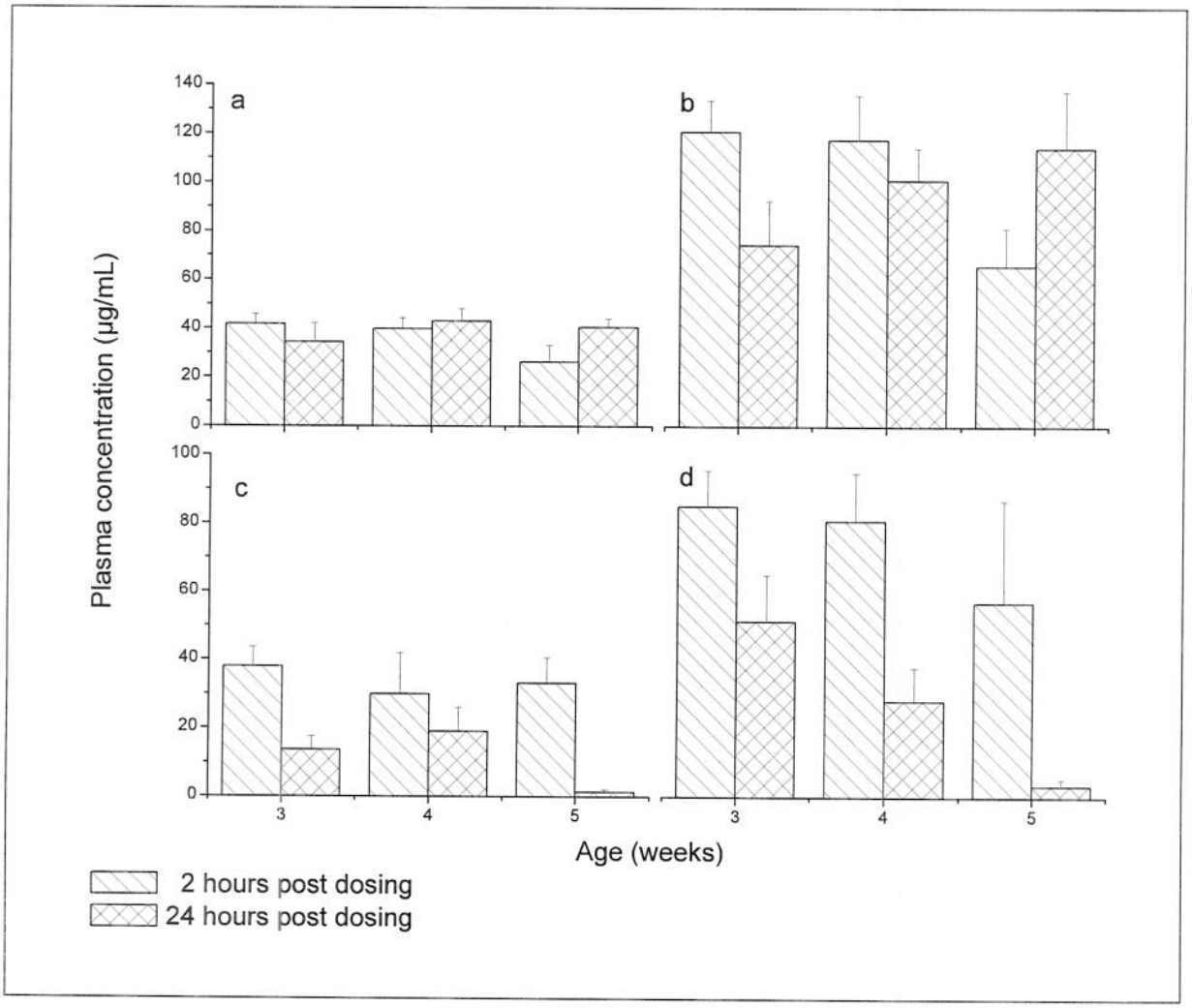
Table 2: Plasma and urine PFOA concentrations in female rats dosed via oral gavage with 10 or 30 mg/kg PFOA

Data expressed as µg/mL

			Plasma				Urine	
	Age	Dose	2 Hours Post-Dose		24 Hours Post-Dose		24 Hours Post-Dose	
Group	(weeks)	(mg/kg)	Mean	SD	Mean	SD	Mean	SD
I	3	10	37.87	5.77	13.55	3.83	21.17	8.95
V	4	10	29.88	12.15	18.98	7.01	23.26	15.27
IX	5	10	33.23	7.41	1.36	0.87	49.77	24.64
III	3	30	84.86	10.51	51.43	13.61	94.89	26.26
VII	4	30	80.67	14.10	28.01	9.90	104.12	28.97
XI	5	30	56.90	29.66	3.42	1.95	123.16	51.56

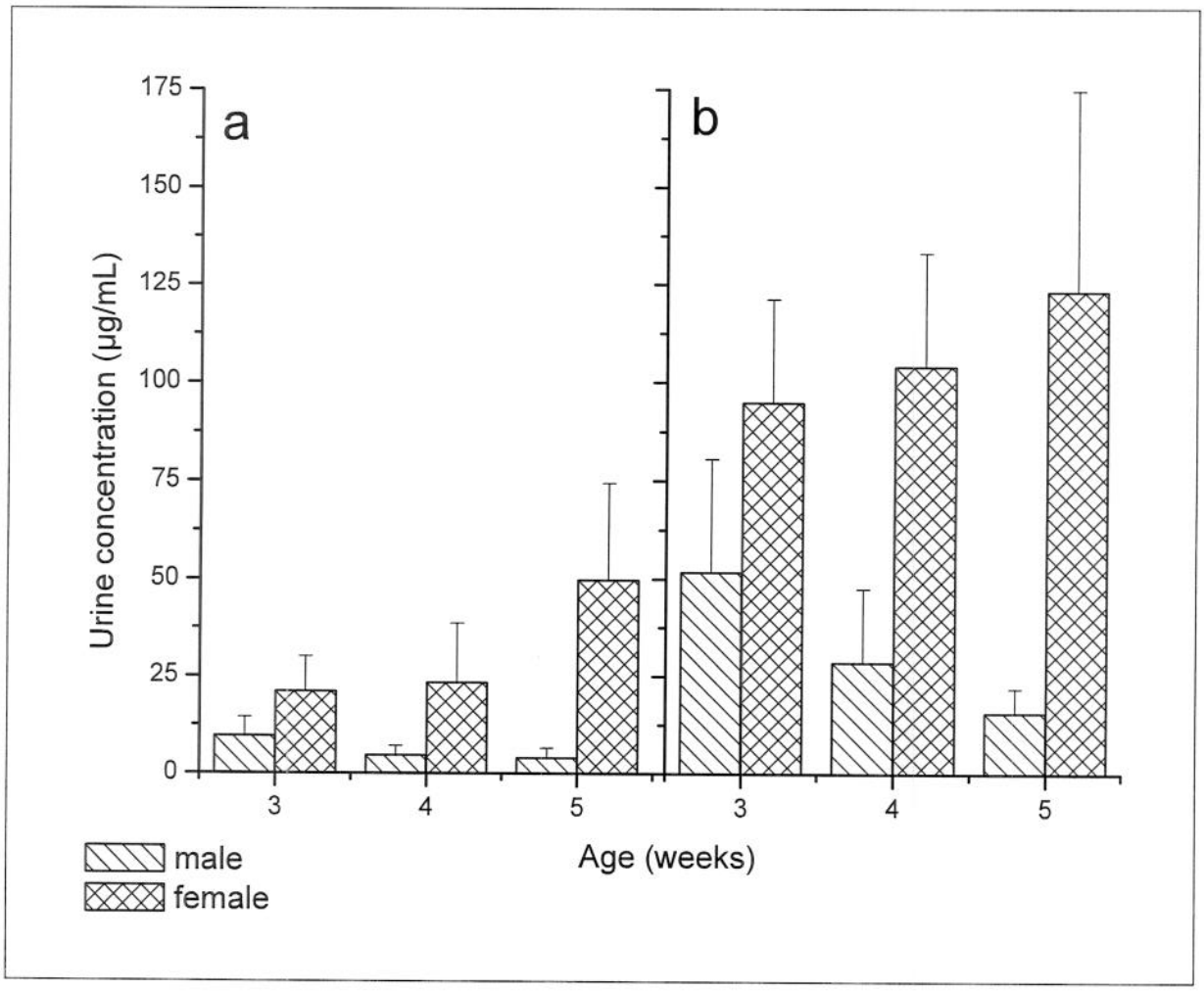
FIGURES

Figure 1: Plasma PFOA concentration in rats following oral gavage with PFOA



a Male plasma at 10 mg/kg (groups I, V and IX).
b Male plasma at 30 mg/kg (groups III, VII and XI).
c Female plasma at 10 mg/kg (groups II, VI and X).
d Female plasma at 30 mg/kg (groups IV, VIII and XII).

Figure 2: Urine PFOA concentration in rats following oral gavage with PFOA



a 10 mg/kg (groups I, II, V, VI, IX and X).
b 30 mg/kg (groups III, IV, VII, VIII, XI and XII).

APPENDICES

APPENDICES

EXPLANATORY NOTES

ABBREVIATIONS:

LOQ	limit of quantitation
SD	standard deviation
SE	standard error
% CV	percent coefficient of variation

Appendix A

Dosing Solution Analysis

Ammonium Perfluorooctanoate: Age Effect on the Plasma Concentration in Post-Weaning Rats Following Oral Gavage

ANALYSIS FOR H-24921 IN DOSING SOLUTIONS

Medical Research Project Number:	15339
Haskell Sample Number:	24921
Analytical Report Number:	DuPont-15302_an

SUMMARY

Dosing solutions at concentrations of 2.5, and 7.5 mg/mL of H-24921 were prepared and collected for uniformity of mixing/concentration verification and stability analysis (5-hour room temperature) on August 9, 2004. In addition, 0 mg/mL (control) sample was submitted for analysis with the set of samples.

Concentrations of H-24921 in separate dosing solutions were measured by high performance liquid chromatography tandem mass spectrometry (LC-MS/MS).

The suspension vehicle for the study was Nanopure ® water.

The results for H-24921 samples prepared and collected on August 9, 2004 indicated that the test substance was at the targeted concentrations (± 16.9% of nominal), adequately mixed (CV less than 10) and stable in the vehicle when held 5 hours at room temperature for all levels.

H-25425 was not detected in the 0 mg/mL (control) samples.

SAMPLE SUBMITTAL

Dosing solutions at concentrations of 2.5, and 7.5 mg/mL of H-24921 were prepared and collected for uniformity of mixing/concentration verification and stability analysis (5-hour room temperature) on August 9, 2004. In addition, 0 mg/mL (control) sample was submitted for analysis with the set of samples.

Samples submitted for analysis were analyzed the day they were received and/or when re-analysis was indicated.

The suspension vehicle for the study was Nanopure® water.

METHODS

1. Analytical Methods

a. Recovery Sample Analysis

Concurrent with dosing solutions analyses, recovery of H-24921 from spiked vehicle (Nanopure® water) was tested at the low level (2.5 mg/mL) and at the high level (7.5 mg/mL) to confirm the analytical method. A stock solution of H-24921 was prepared in Nanopure® water. For all concentration levels, an appropriate aliquot of this solution to obtain approximately 1.25 mg (low), and 3.75 mg (high) of the test material was added to 100 mL of Nanopure® water. All recovery samples were then mixed for dispersion of the test material in the vehicle. The samples were then processed and analyzed in the same manner as the dosing samples at similar concentrations.

b. Dosing Suspension Treatment

Each dosing sample (0.5 mL) was diluted to 100 mL with Nanopure® water and mixed to dissolve the H-24921 in the suspension. The dosing samples were further diluted with Nanopure® water to an expected concentration of approximately 0.00003 mg/mL prior to analysis. Before all final dilutions, the internal standard and the 0 mg/mL sample (initial dilution) was added to each test sample to give an equivalent final concentration of the internal standard and the matrix in all samples.

Samples submitted for analysis were analyzed the day the solutions were received and /or when re-analysis was indicated.

c. Chromatographic Conditions

LC parameters

Instrument:	Water Alliance 2795 HPLC
Column:	Zorbax® RX-C8, 2.1 mm x 150 mm, 5μm
Flow Rate:	0.4 mL/min
Injection Volume:	20 μl
Column Temperature:	35°C
Column Switch:	5.0 minutes
Mobile Phase:	0.15% Acetic Acid/Acetonitrile
Gradient:	

Time (min.)	% Acetonitrile
0.0	5
0.9	5
1.0	80
5.0	80
5.1	5
8.5	5

MS parameters

Instrument:	Micromass Quattro Micro Tandem MS
Ionization mode:	Electrospray (ESI), negative ion
Capillary voltage:	2.7 kV
Cone Voltage:	15 V
Source Temperature:	120°
Desolvation Temperature:	350°
Scan function:	PFOA: 413 m/z (parent) to 369 m/z (daughter) 1,2-di-^{13}C PFOA: 415 m/z (parent) to 370 m/z (daughter)

Retention Time of PFOA and 1,2-di-^{13}C PFOA: approximately 3.5 minutes (20 μL injections)

d. Calibration and Quantitation

A stock solution of Analytical Standard (APFO, H-22703-265, 100% pure) was made in acetonitrile. Appropriate aliquots of the stock were diluted with Nanopure® water to make calibration standards that bracketed the target concentration of the diluted sample solutions. Before these aliquot were brought to volume, an appropriate amount of internal standard (1,2-di-^{13}C perfluorooctanoic acid) was added.

Analysis of H-24921 was by high-performance liquid chromatography/tandem mass spectrometry (LC-MS/MS). Negative-ion electrospray is used to generate negatively charged ions of PFOA, the ions are selected with the first MS quadrupole, collisionally dissociated using argon, and a fragment ion is monitored. 1,2-di-^{13}C perfluorooctanoic acid is used as an internal standard. Triplicate injection of the sample solutions and calibration standards solutions were made and peak areas were calculated electronically.

The calibration curve was generated by regression analysis using the peak area ratio from the analytical standard and the internal standard (refer to Figure 1). Data for test solutions were compared to the calibration curves to evaluate the concentrations of the H-24921.

Test substance uniformity in the vehicle was evaluated by calculating the coefficient of variation (C.V. = standard deviation/mean x 100) of the measured concentrations of the duplicate samples (uniformity of mixing/concentration verification) for each dosing level. A coefficient of variation of less than or equal to 10% is the standard criterion at Haskell Laboratory for acceptable distribution of the test substance throughout the solution.

The mean result of the concentration verification samples (n = 2) for each dosing level was used to determine the concentration of the test substance for the respective dosing levels.

Stability was evaluated by using the mean result of the uniformity of mixing/concentration verification samples as the baseline for comparing the corresponding stability results.

ANALYTICAL RESULTS

A. Chromatography

H-24921 eluted from the HPLC column as a resolved peak with a retention time of approximately 3.5 minutes (20 µL injections) for the negative ion. Representative LC-MS/MS chromatograms are shown in Figures 2 (a - f). Test substance was not detected in the 0 mg/mL control. This was determined by a comparison of the diluent water with and without internal standard against the 0 mg/mL control containing internal standard. Refer to Figures 2 (a - c).

B. Recovery Samples

Detailed analytical results of recovery samples are summarized in Table I. The measured concentration of H-24921 for the 2.5 mg/mL level was 97.3% of nominal. The measured concentration of H-24921 for the 7.5 mg/mL level was 102.1% of nominal. Based on this data, the analytical method performed satisfactorily over the entire concentration range for the study.

C. Uniformity of Mixing/Concentration and Stability Samples

Analytical results from dosing solutions collected on August 9, 2004 and analyzed for uniformity of mixing/concentration verification and 5-hour room temperature stability are shown in Appendix <>, Table II and Summary Table 1.

The following table summarizes the results for uniformity/concentration verification and stability analyses for this sampling day of the H-24921 preparation.

Sample Day Sample Type	Nominal mg/mL	Measured [a] mg/mL	Mean % Nominal	C.V. (%)	Stability[b] % Nominal
9-Aug-2004					
Uniformity/Concentration	0	ND[c]	---	---	---
	2.5	2.98, 2.80	115.5	4	118.4
	7.5	8.65, 8.88	116.9	2	119.0

a Mean results for the analysis of the duplicate samples.
b Samples held 5 hours at room temperature.
c Denotes none detected. Reported results are based on comparison of the diluent water with and without the internal standard addition and the control with the internal standard addition. Shown in Figures 2 (a - c).

The results for H-24921 samples prepared and collected on August 9, 2004 indicated that the test substance was at the targeted concentrations (± 16.9% of nominal), adequately mixed (CV less than 10) and stable in the vehicle when held 5 hours at room temperature for all levels. Test substance was not detected in the 0 mg/mL samples.

E. Conclusion

Results from the analysis of the H-24921 dosing solutions for the study indicate that the test substance was mixed properly (CV less than 10), at the targeted levels (± 20% of nominal) and stable under the conditions of the study. Test substance was not found in the 0 mg/mL samples.

Table I. Recovery of H-24921 Added to Dosing Vehicle

Sample Type	mg/ml H-24921 Nominal	Measured	Percent Nominal
RECOVERY[A]	2.52	2.45[B]	97.3
RECOVERY[A]	7.45	7.61	102.1

[A] Processed with uniformity of mixing/concentration verification samples from dosing prepared on August 9, 2004.

[B] Reported result is from a duplicate preparation of the recovery sample. The original recovery sample analyzed higher than expected due to aliquot error and is not reported..

Table II. Uniformity of Mixing/Concentration Verification and Stability of H-24921 in Dosing Solutions

Sample Type	mg/mL H-24921 Nominal	Measured	Percent Nominal
9-August-2004			
Uniformity/Concentration			
CONTROL	0.00	ND[A]	---
#1	2.5	2.98	119.1
#2	2.5	2.80	111.8
		Mean[B]***: 2.89 ± 0.13***	***(115.5%)***
		C.V. 4%	
#1	7.5	8.65	115.3
#2	7.5	8.88	118.4
		Mean[B]***: 8.77 ± 0.16***	***(116.9%)***
		C.V. 2%	
5 HOUR[C]	2.5	2.96	118.4
	7.5	8.93	119.0

[A] Denotes none detected. Reported results are based on comparison of the diluent water with and without the internal standard addition and the control with the internal standard addition. Shown in Figures 2 (a - c).

[B] The average measured concentration, average percent of nominal (in parentheses), standard deviation, and coefficient of variation of duplicate samples.

[C] Stability samples held 5 hours a room temperature.

Figure 1
Representative Analytical Calibration Curves

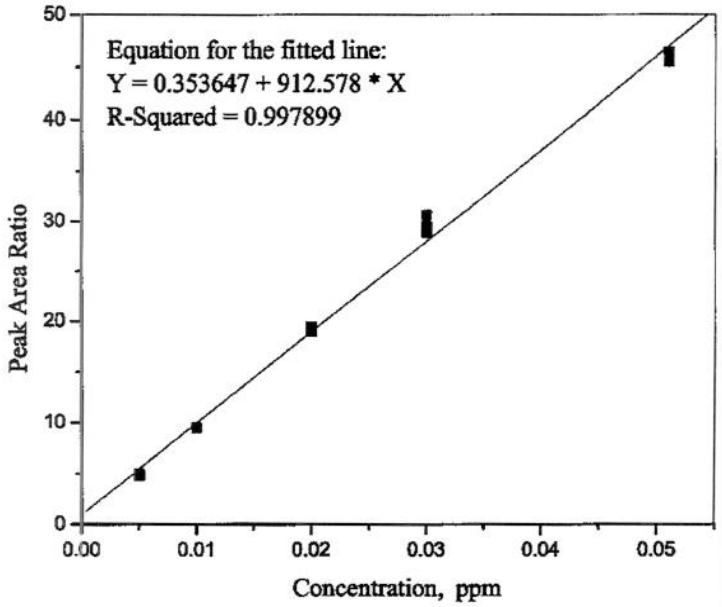


Figure 1: Calibration curve showing linear fit (line) to replicate peak area ratio (squares) for calibration solutions of H-22703-265 (APFO Analytical Standard) diluted with Nanopure® water and matrix matched over a concentration range of 0.005 to 0.051 ppm.

Figure 2

Representative LC/MS/MS Chromatography Chromatograms

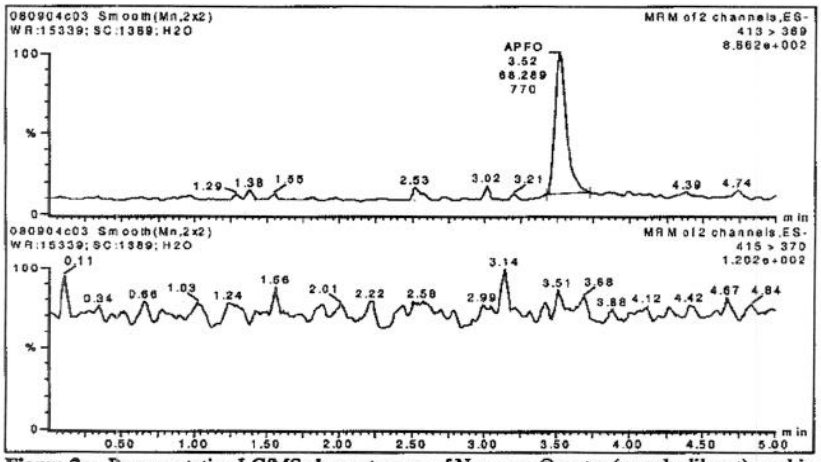


Figure 2a: Representative LC/MS chromatogram of Nanopure® water (sample diluent) used in the study. Negative ion/PFOA retention time is approximately 3.5 minutes.

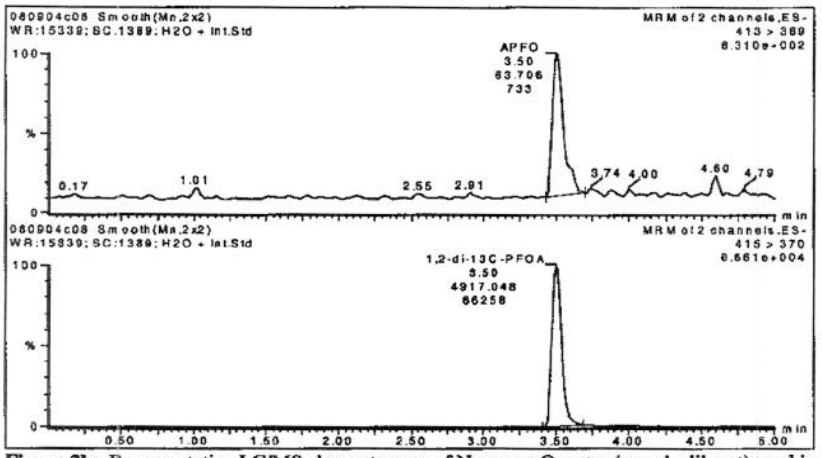


Figure 2b: Representative LC/MS chromatogram of Nanopure® water (sample diluent) used in the study with the internal standard. Negative ion/PFOA retention time is approximately 3.5 minutes.

Figure 2 (continued)

Representative LC/MS/MS Chromatography Chromatograms

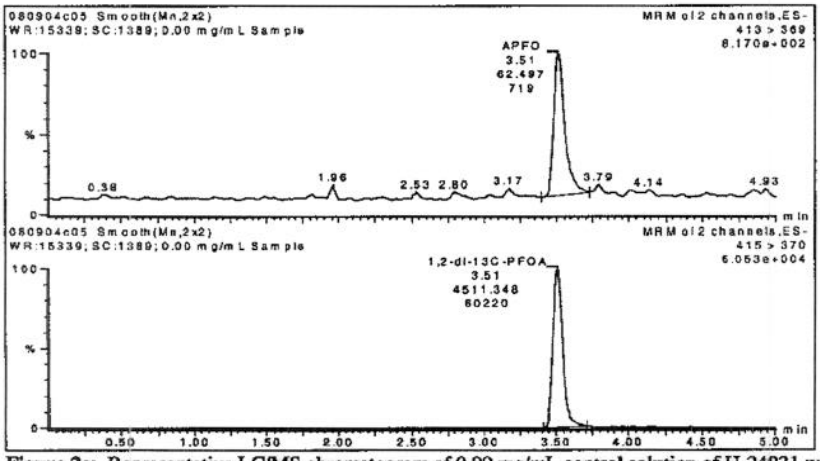


Figure 2c: Representative LC/MS chromatogram of 0.00 mg/mL control solution of H-24921 with the internal standard. Negative ion/PFOA retention time is approximately 3.5 minutes.

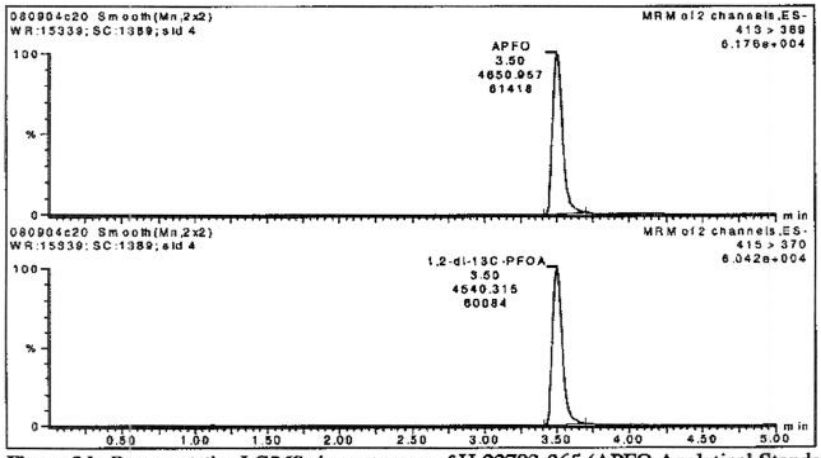


Figure 2d: Representative LC/MS chromatogram of H-22703-265 (APFO Analytical Standard) (0.030 ppm) with the internal standard (0.0315 ppm). Negative ion/PFOA retention time is approximately 3.5 minutes.

Figure 2 (continued)

Representative LC/MS/MS Chromatography Chromatograms

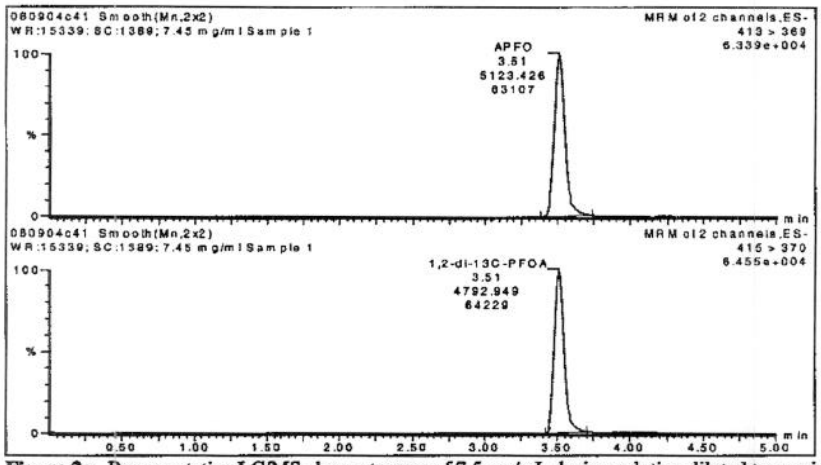


Figure 2e: Representative LC/MS chromatogram of 7.5 mg/mL dosing solution diluted to nominal concentration of 0.03 ppm of H-24921 for negative ion /PFOA with retention time 3.5 minutes. The measured concentration of the representative solution is 8.65 mg/mL.

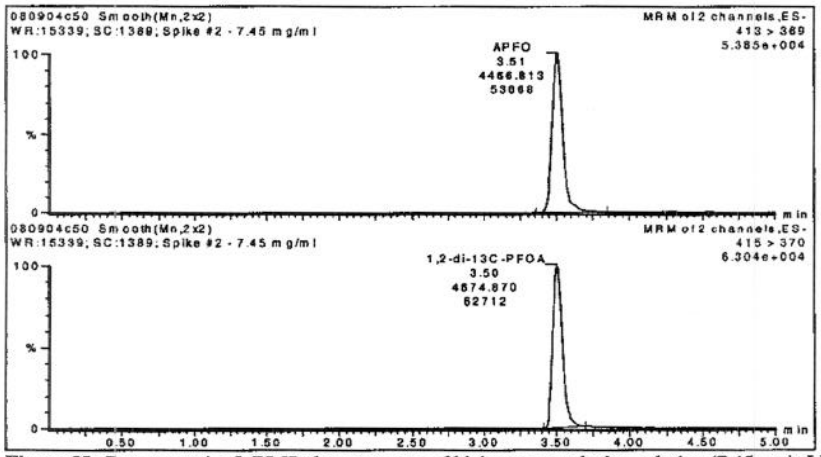


Figure 2f: Representative LC/MS chromatogram of high recovery dosing solution (7.45 mg/mL) diluted to nominal concentration of 0.03 ppm of H-24921 for negative ion /PFOA with retention time approximately 3.5 minutes. The measured concentration of the representative recovery solution is 7.61 mg/mL.

Table 1
Summary of Dosing Suspension Analyses

Sample Type	Dosing Concentrations and Stability of H-24921 (mg/mL)	
Nominal:	2.5	7.5
Uniformity/Concentration		
August 9, 2004		
control	0.0	ND [a]
#1	2.98	8.65
	(119.1) [b]	(115.3)
#2	2.80	8.88
	(111.8)	(118.4)
Average Measured Conc.[c]	**2.89**	**8.77**
Average Percent Nominal[c]	**(115.5)**	**(116.9)**
Standard Deviation[c]	**± 0.13**	**± 0.16**
Coefficient of Variation[c]	**4%**	**2%**
Stability		
5-hour Room Temperature	2.96	8.93
	(118.4)	(119.0)

[a] Denotes none detected. Reported results are based on comparison of the diluent water with and without the internal standard addition and the control with the internal standard addition. Shown in Figures 2 (a - c).

[b] Numbers in parentheses are the respective percent of nominal values.

[c] The average measured concentration, average percent of nominal (in parentheses), standard deviation, and coefficient of variation of duplicate samples.

Appendix B

PFOA Plasma Concentration Data - Males

Group	Age (weeks)	Time Post Dose (hours)	Animal Number	Dose (mg/kg)	PFOA Plasma Concentration (µg/mL)	Mean	SD
I	3	2	1	10	47.20	41.87	4.01
I	3	2	2	10	44.30		
I	3	2	3	10	39.80		
I	3	2	4	10	41.19		
I	3	2	6	10	36.85		
III	3	2	8	30	138.95	120.65	12.78
III	3	2	10	30	123.62		
III	3	2	12	30	104.66		
III	3	2	15	30	113.53		
III	3	2	16	30	122.48		
V	4	2	51	10	35.37	39.92	4.45
V	4	2	53	10	43.13		
V	4	2	54	10	35.85		
V	4	2	58	10	45.54		
V	4	2	61	10	39.70		
VII	4	2	52	30	117.29	117.40	18.10
VII	4	2	55	30	86.41		
VII	4	2	56	30	131.53		
VII	4	2	57	30	127.73		
VII	4	2	60	30	124.03		
IX	5	2	99	10	29.36	26.32	6.89
IX	5	2	100	10	37.00		
IX	5	2	103	10	21.75		
IX	5	2	105	10	23.09		
IX	5	2	107	10	20.39		
XI	5	2	96	30	59.63	65.66	15.53
XI	5	2	97	30	49.51		
XI	5	2	98	30	58.10		
XI	5	2	101	30	71.36		
XI	5	2	102	30	89.69		

Group	Age (weeks)	Time Post Dose (hours)	Animal Number	Dose (mg/kg)	PFOA Plasma Concentration (µg/mL)	Mean	SD
I	3	24	9	10	35.23	34.22	7.89
I	3	24	13	10	21.00		
I	3	24	14	10	36.13		
I	3	24	18	10	42.23		
I	3	24	24	10	36.49		
III	3	24	17	30	65.30	74.16	18.23
III	3	24	19	30	76.80		
III	3	24	21	30	80.05		
III	3	24	22	30	98.90		
III	3	24	25	30	49.73		
V	4	24	62	10	40.04	42.94	5.33
V	4	24	63	10	42.68		
V	4	24	65	10	47.32		
V	4	24	66	10	48.85		
V	4	24	70	10	35.80		
VII	4	24	64	30	112.17	100.81	13.18
VII	4	24	67	30	106.94		
VII	4	24	68	30	108.70		
VII	4	24	71	30	96.60		
VII	4	24	72	30	79.64		
IX	5	24	108	10	37.05	40.60	3.69
IX	5	24	109	10	39.60		
IX	5	24	110	10	40.52		
IX	5	24	115	10	39.05		
IX	5	24	116	10	46.80		
XI	5	24	106	30	98.30	113.86	23.36
XI	5	24	111	30	98.70		
XI	5	24	112	30	103.53		
XI	5	24	113	30	153.89		
XI	5	24	114	30	114.88		

Appendix C

PFOA Plasma Concentration Data - Females

Group	Age (weeks)	Time Post Dose (hours)	Animal Number	Dose (mg/kg)	PFOA Plasma Concentration (µg/mL)	Mean	SD
II	3	2	26	10	29.64	37.87	5.77
II	3	2	27	10	37.87		
II	3	2	31	10	45.90		
II	3	2	33	10	38.63		
II	3	2	38	10	37.32		
IV	3	2	28	30	96.93	84.86	10.51
IV	3	2	30	30	89.64		
IV	3	2	32	30	74.41		
IV	3	2	35	30	90.13		
IV	3	2	36	30	73.18		
VI	4	2	73	10	27.69	29.88	12.15
VI	4	2	74	10	31.05		
VI	4	2	75	10	19.27		
VI	4	2	77	10	49.91		
VI	4	2	78	10	21.48		
VIII	4	2	76	30	103.54	80.67	14.10
VIII	4	2	80	30	67.98		
VIII	4	2	82	30	70.38		
VIII	4	2	83	30	79.15		
VIII	4	2	84	30	82.28		
X	5	2	122	10	44.00	33.23	7.41
X	5	2	124	10	23.66		
X	5	2	126	10	33.88		
X	5	2	127	10	30.08		
X	5	2	128	10	34.51		
XII	5	2	117	30	78.17	56.90	29.66
XII	5	2	118	30	98.61		
XII	5	2	120	30	35.55		
XII	5	2	121	30	34.86		
XII	5	2	125	30	37.30		

Group	Age (weeks)	Time Post Dose (hours)	Animal Number	Dose (mg/kg)	PFOA Plasma Concentration (µg/mL)	Mean	SD
II	3	24	40	10	8.82	13.55	3.83
II	3	24	44	10	13.70		
II	3	24	46	10	13.76		
II	3	24	49	10	19.38		
II	3	24	50	10	12.08		
IV	3	24	37	30	56.25	51.43	13.61
IV	3	24	39	30	51.05		
IV	3	24	42	30	42.29		
IV	3	24	43	30	71.43		
IV	3	24	45	30	36.13		
VI	4	24	79	10	16.63	18.98	7.01
VI	4	24	81	10	23.20		
VI	4	24	89	10	24.04		
VI	4	24	91	10	7.67		
VI	4	24	94	10	23.39		
VIII	4	24	86	30	37.70	28.01	9.90
VIII	4	24	87	30	39.31		
VIII	4	24	88	30	21.62		
VIII	4	24	90	30	17.32		
VIII	4	24	92	30	24.09		
X	5	24	130	10	2.49	1.36	0.87
X	5	24	131	10	0.92		
X	5	24	132	10	0.36		
X	5	24	133	10	1.00		
X	5	24	137	10	2.02		
XII	5	24	129	30	0.61	3.42	1.95
XII	5	24	134	30	5.53		
XII	5	24	135	30	4.33		
XII	5	24	136	30	4.33		
XII	5	24	138	30	2.30		

Appendix D

Supplemental PFOA Plasma Concentrations

Animal	PFOA Plasma Concentration (µg/mL)	Mean	SD
Fasted Male 1	84.28	64.95	27.34
Fasted Male 2	45.62		
Fasted Female 1	58.20	68.16	14.10
Fasted Female 2	78.13		
Non-Fasted Male 3	29.81	30.00	0.28
Non-Fasted Male 4	30.20		
Non-Fasted Female 3	31.94	26.54	7.64
Non-Fasted Female 4	21.14		

Note: All animals were 3 weeks of age and received a single 10 mg/kg dose. Plasma samples were collected 24 hours post-dosing.

Appendix E

Urine PFOA Concentration Data - Males

Group	Age (weeks)	Animal Number	Dose (mg/kg)	PFOA Urine Concentration (µg/mL)	Mean	SD
I	3	9	10	9.26	9.57	4.86
I	3	13	10	16.21		
I	3	14	10	3.41		
I	3	18	10	11.97		
I	3	24	10	6.97		
III	3	17	30	23.69	51.76	28.86
III	3	19	30	71.90		
III	3	21	30	80.97		
III	3	22	30	NS		
III	3	25	30	30.48		
V	4	62	10	0.72	4.53	2.45
V	4	63	10	3.62		
V	4	65	10	5.45		
V	4	66	10	5.85		
V	4	70	10	7.01		
VII	4	64	30	16.32	28.70	18.84
VII	4	67	30	22.55		
VII	4	68	30	10.99		
VII	4	71	30	58.17		
VII	4	72	30	35.47		
IX	5	108	10	2.78	4.03	2.36
IX	5	109	10	4.04		
IX	5	110	10	2.35		
IX	5	115	10	2.85		
IX	5	116	10	8.10		
XI	5	106	30	11.24	15.65	6.24
XI	5	111	30	13.75		
XI	5	112	30	25.61		
XI	5	113	30	17.46		
XI	5	114	30	10.16		

NS = no urine sample was produced by the animal.

Appendix F

Urine PFOA Concentration Data - Females

Group	Age (weeks)	Animal Number	Dose (mg/kg)	PFOA Urine Concentration (µg/mL)	Mean	SD
II	3	40	10	17.59	21.17	8.95
II	3	44	10	19.36		
II	3	46	10	10.04		
II	3	49	10	24.73		
II	3	50	10	34.11		
IV	3	37	30	116.09	94.89	26.36
IV	3	39	30	83.67		
IV	3	42	30	95.85		
IV	3	43	30	56.68		
IV	3	45	30	122.17		
VI	4	79	10	23.21	23.26	15.27
VI	4	81	10	7.16		
VI	4	89	10	24.79		
VI	4	91	10	47.37		
VI	4	94	10	13.79		
VIII	4	86	30	125.55	104.12	28.97
VIII	4	87	30	60.35		
VIII	4	88	30	90.84		
VIII	4	90	30	131.16		
VIII	4	92	30	112.68		
X	5	130	10	41.04	49.77	24.64
X	5	131	10	23.85		
X	5	132	10	89.76		
X	5	133	10	52.89		
X	5	137	10	41.31		
XII	5	129	30	99.53	123.16	51.56
XII	5	134	30	111.16		
XII	5	135	30	55.41		
XII	5	136	30	175.44		
XII	5	138	30	174.25		