

Study Title

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

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STUDY COMPLETED ON: December 15, 2003

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
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LABORATORY PROJECT ID: DuPont-13267

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards.

Study Director: _____


King Han, Ph.D.
Research Toxicologist
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15-Dec-2003
Date

QUALITY ASSURANCE STATEMENT

Haskell Sample Number(s):

24921

Dates of Inspections:

Protocol: June 18, 2003

Conduct: July 8,9,21,28,30, 2003

Records, Reports: October 27-30, 2003

Dates Findings Reported to:

Study Director: June 18, 2003; November 3,11, 2003

Management: November 3,11, 2003

Reported by:

Wonda K. Kelly

Wonda K. Kelly
Quality Assurance Auditor

15-Dec-2003
Date

CERTIFICATION

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

Approved by: *W. Jepson for Gary Jepson* 12-Dec-2003
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Principal Research Toxicologist and Manager

Approved by: *Matthew S. Bogdanffy* 12-Dec-2003
Matthew S. Bogdanffy, Ph.D., D.A.B.T. Date
Research Manager and Director

Issued by Study Director: *Xing Han* 15-Dec-2003
Xing Han, Ph.D. Date
Research Toxicologist

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STUDY INFORMATION

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

Synonyms/Codes: • Ammonium perfluorooctanoate

[REDACTED]

- C-8
- Perfluorooctanoate, ammonium salt
- PFOA
- H-24921

[REDACTED]

Haskell Number: 24921

CAS Registry Number: [REDACTED]

Purity: [REDACTED]

Known Impurities: [REDACTED]

Physical Characteristics: White solid

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

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Experimental Start/Completion: June 30, 2003 / August 7, 2003

Study Initiated/Completed: June 27, 2003 / (see report cover page)

STUDY PERSONNEL

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SUMMARY

The relationship between age and plasma concentration of ammonium perfluorooctanoate (PFOA) in rats has been investigated in this study. Male and female rats between 4 and 8 weeks old were administered 10 mg/kg of PFOA by single oral gavage. The plasma concentration of PFOA at 24 hours post-dose was measured by LC/MS. The results showed that the plasma concentration of PFOA in rats was dependent on the age and sex of the rats.

A. Age Effect

In both male and female rats, the greatest age-related difference in PFOA plasma concentration was observed between 4 and 5 weeks of age. The 5-week old male rats had PFOA plasma concentrations that were 5.4-fold higher than the 4-week old male rats. In contrast, the PFOA plasma concentrations in the 5-week old female rats were 2.4-fold lower than in the 4-week old rats. PFOA levels in plasma were no longer significantly affected by age in rats of either sex that were older than 5 weeks (through study termination at 8 weeks).

B. Sex Comparison

At 4 weeks of age, male rats had PFOA plasma concentrations that were 2.7-fold higher than in females. The PFOA plasma concentrations in 5- to 8-week old male rats were 34.7- to 65.1-fold higher than that in the females of the same age.

INTRODUCTION

Ammonium perfluorooctanoate (PFOA) is a perfluorinated octanoic acid salt used as an industrial surfactant. Elimination of PFOA in rats is sex-dependent and hormone regulated. PFOA elimination is much faster in female rats than in male rats,⁽¹⁾ down-regulated by testosterone in both female and castrated male rats,^(2,3) and up-regulated by estradiol in the male rats.⁽⁴⁾ However, these studies were conducted on sexually mature rats. In the current study, male and female post-weaning rats at different ages were dosed with PFOA, and the plasma concentrations of PFOA at 24 hours post-dosing were compared. This post-dosing time point was chosen because 24 hours after dosing, the sex difference in PFOA elimination in mature rats can be easily distinguished via the plasma concentration of PFOA.⁽⁵⁾ The objective of this study was to determine if immature male and female rats eliminate PFOA from blood at rates different than those observed in mature rats. This study was not designed to fully characterize the pharmacokinetics of PFOA in immature rats, and was not designed for use in determining definitive elimination rate constants.

MATERIALS AND METHODS

A. Test Substance

PFOA (ammonium perfluorooctanoate [REDACTED]) 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) was provided by the vendor and documented in the study records and report.

CAS Registry Number: [REDACTED]

Molecular Weight: 414.1

Molecular Formula: [REDACTED]

B. Test System

Male and female Crl:CD®(SD)IGSBR rats were obtained from Charles River Laboratories, Inc., Raleigh, North Carolina. 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.^(2,5,6)

At the time of dosing, rats were 4, 5, 6, 7, and 8 weeks of age and the weight variation did not exceed $\pm 20\%$ 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.

C. Animal Husbandry

1. Animal Housing

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

Throughout the dosing period with test substance, the rats were housed individually in appropriate cages according to the SOP.

2. Environmental Conditions

Animal room(s) 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 was recorded but not included in the final report.

3. Feed and Water

All animals were provided tap water *ad libitum* and fed PMI® Nutrition International, LLC Certified Rodent LabDiet® 5002 *ad libitum*. Animals were fasted overnight for approximately 12 hours before dosing with test substance. Food was returned approximately two hours post-dose.

4. Animal Health and Environmental Monitoring Program

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

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

Certified animal feed is 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 would not be expected to impact the integrity of the study.

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

D. In-Life Methods

1. Animals

The experimental groups were as shown below:

Group	Weeks	Age*		Number of Rats	
		Days	Male	Female	
I	4	28	10	10	
II	5	35	10	10	
III	6	42	10	10	
IV	7	49	10	10	
V	8	56	10	10	

* Animals were ± 1 day from the target age at the time of dosing. The actual age at dosing is documented in the study records.

2. Dose Preparation, Analysis, and Rates

The test substance was administered to each rat as a single oral gavage. This route was chosen because it is the route most commonly used for toxicity studies of PFOA. PFOA was mixed with HPLC grade water to achieve the target dose concentrations. For all experiments, a single dose level was used equivalent to 10 mg PFOA/kg body weight, and the dose volume was approximately 4 mL/kg body weight. Dose solutions were prepared one day before the day of use and stored in a 4°C refrigerator prior to use.

LC/MS methods that were used for dose verification are described in Section E, Analytical Methods.

3. Sacrifice and Blood Sampling

Rats were sacrificed by CO₂ asphyxiation and exsanguinated by cardiac puncture.

4. Experimental Procedure

Rats were administered PFOA as a single oral dose of 10 mg/kg. Twenty-four hours after PFOA administration, rats were sacrificed and whole blood samples were collected by cardiac puncture. Blood samples were all collected within 20 minutes of the desired sample time. Whole blood samples were placed into EDTA tubes and maintained on wet ice. Plasma was separated by centrifugation and frozen at $<-10^{\circ}\text{C}$ until analysis.

Kidneys and livers were collected at sacrifice and were flash frozen in liquid nitrogen and stored at -60°C for possible future biochemical analysis.

E. Analytical Methods

1. Verification of Dose Solution

PFOA standards (1.0, 0.5, 0.2, 0.1, and 0.05 $\mu\text{g/mL}$) were prepared in HPLC-grade water. The dose solution was diluted by 10,000-fold prior to the analysis by LC/MS method.

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 $\mu\text{g/mL}$ 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 1:4 (60 μL of internal standard solution) to 1:50 (980 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 2790 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):	0
RF lens (V):	0
Source temperature:	130 $^{\circ}\text{C}$
Desolvation temperature:	350 $^{\circ}\text{C}$
MS Method:	
Mode:	MRM, 2 transitions
Time:	0-10.0 min
Ch1:	413.00 $\rightarrow$ 369.00
Dwell:	0.25 sec

Ch2: Collision energy: 15 eV
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 μ m
Column temperature: Ambient
Mobile phases: A: 50 mM Ammonium Acetate
B: Acetonitrile

Gradient:

Time (min)	%B
0.0	10
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 μ L

F. Statistical Analysis

Group data is represented by mean, standard deviation, standard error, and percent coefficient of variation. Statistical significance was assessed by the Student's t-test. Rejection of data is based on Chauvenet's criterion.⁽⁷⁾

RESULTS AND DISCUSSION

A. Dose Verification (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 E. 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 mg/mL, Appendix A).

B. Plasma Concentration of PFOA
(Table 1, Figures 1-3, Appendix B)

1. Male Rats

The plasma concentrations of PFOA in the male rats are shown in Table 1. Their relationship with the age of the male rats is plotted in Figure 1. The PFOA plasma concentration was $7.32 \pm 1.01 \mu\text{g/mL}$ (mean $\pm$ SD) in 4-weeks old male rats. In contrast, a higher PFOA concentration ($39.24 \pm 3.89 \mu\text{g/mL}$) was observed in the plasma of 5-week old male rats. The mean PFOA plasma concentrations in 6-, 7-, and 8-week old male rats were in a range of 37.12 to $43.19 \mu\text{g/mL}$, which, based on the results of unpaired t-test at 99% confidence interval, were not significantly different from the plasma concentration of PFOA in the 5-week old male rats.

2. Female Rats

The plasma concentrations of PFOA in the female rats are shown in Table 1. Their relationship with the age of the female rats is plotted in Figure 2. The plasma concentration of PFOA in 4-week old female rats was $2.68 \pm 0.64 \mu\text{g/mL}$, which was significantly higher ($p < 0.01$) than the PFOA concentrations (mean ranged from 0.57 to $1.18 \mu\text{g/mL}$) in the elder females that were 5 to 8 weeks old.

3. Comparison between Male and Female Rats

PFOA plasma concentrations in male and female rats are shown in Figure 3. PFOA plasma concentrations were higher in the males than in the females for all age groups. The most dramatic sex-dependent difference in PFOA concentration was observed for rats that were 5 weeks and older. The ratios of PFOA concentrations between male and female rats that were 5 to 8 weeks old were all greater than 34, whereas the ratio was only 2.7 for the 4-week old rats.

CONCLUSIONS

Age has a significant effect on the plasma concentrations of PFOA in both male and female rats following oral dosing with 10 mg/kg PFOA. The sex-related difference in PFOA plasma concentration is most pronounced for rats that are older than 5 weeks, with male rats having PFOA plasma levels more than 34-fold higher than female rats.

RECORDS AND SAMPLE STORAGE

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.
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6. 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.
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TABLES

TABLES

EXPLANATORY NOTES

ABBREVIATIONS:

SD standard deviation

Table 1: Plasma concentration in rats 24 hours post-dose following oral gavage with 10 mg/kg PFOA.

Group	Age (weeks)	Males		Females	
		Mean	SD	Mean	SD
I	4	7.32	1.01	2.68	0.64
II	5	39.24	3.89	1.13	0.46
III	6	43.19	3.79	1.18	0.52
IV	7	37.12	4.07	0.57	0.29
V	8	38.55	5.44	0.81*	0.27*

Data expressed as $\mu\text{g/mL}$.

* According to Chauvenet's criterion,⁽⁷⁾ the value of PFOA plasma concentration obtained from rat 511 (2.66 $\mu\text{g/mL}$) was not included for the calculation of the mean and the SD.

FIGURES

Figure 1: Plasma concentration in male rats 24 hours post-dose following oral gavage with 10 mg/kg PFOA.

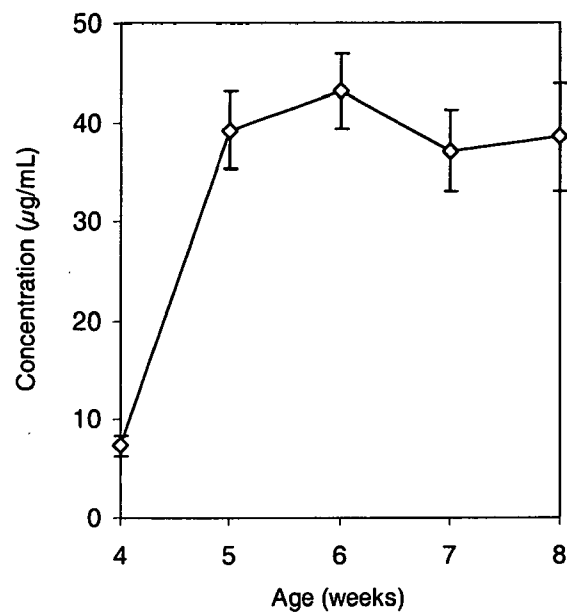


Figure 2: Plasma concentration in female rats 24 hours post-dose following oral gavage with 10 mg/kg PFOA.

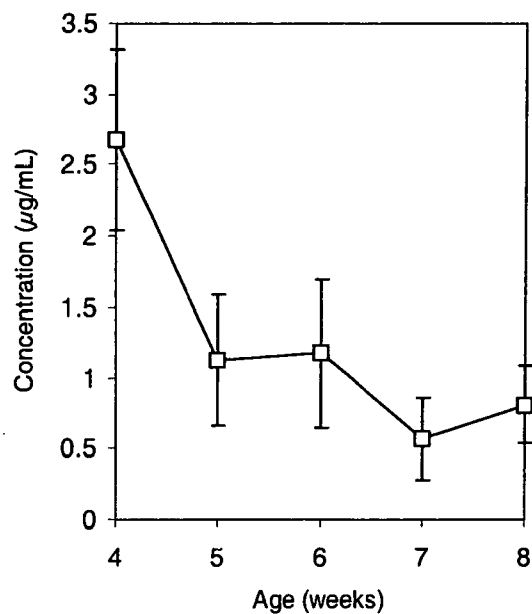
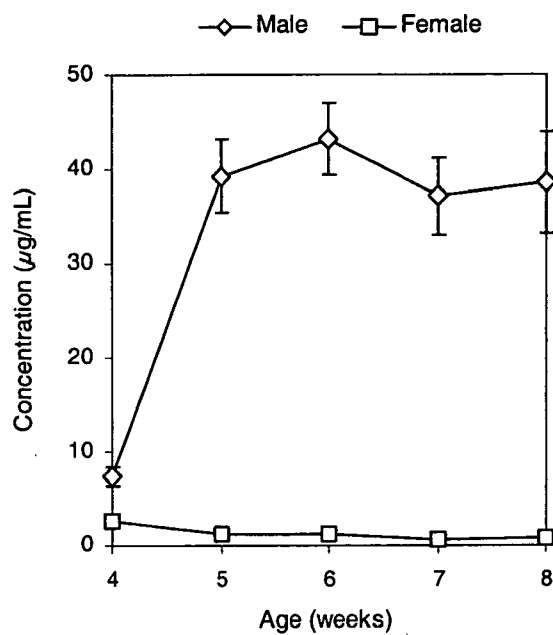


Figure 3: Plasma concentration in male and female rats 24 hours post-dose following oral gavage with 10 mg/kg PFOA.



APPENDICES

APPENDICES

EXPLANATORY NOTES

ABBREVIATIONS:

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

APPENDIX A

Dose Verification Data

Number of Replicates	Description	mg/mL
Dose Solution I – Group I		
1	250 ng/mL dilution – 3A	2.64
2	250 ng/mL dilution – 3A	3.01
3	250 ng/mL dilution – 3A	2.70
4	250 ng/mL dilution – 3B	2.83
5	250 ng/mL dilution – 3B	2.60
6	250 ng/mL dilution – 3B	2.84
7	250 ng/mL dilution – 3C	2.80
8	250 ng/mL dilution – 3C	2.75
9	250 ng/mL dilution – 3C	2.78
Mean		2.77
SD		0.12
SE		0.04
% CV		4.45
Dose Solution II – Group II		
1	250 ng/mL dilution – 3A	2.29
2	250 ng/mL dilution – 3A	2.06
3	250 ng/mL dilution – 3A	1.93
4	250 ng/mL dilution – 3B	2.20
5	250 ng/mL dilution – 3B	2.22
6	250 ng/mL dilution – 3B	1.87
7	250 ng/mL dilution – 3C	1.77
8	250 ng/mL dilution – 3C	1.86
9	250 ng/mL dilution – 3C	1.85
Mean		2.01
SD		0.19
SE		0.06
% CV		9.47
Dose Solution III – Group III		
1	250 ng/mL dilution – 3A	2.69
2	250 ng/mL dilution – 3A	2.65
3	250 ng/mL dilution – 3A	2.62
4	250 ng/mL dilution – 3B	2.59
5	250 ng/mL dilution – 3B	2.82
6	250 ng/mL dilution – 3B	2.55
7	250 ng/mL dilution – 3C	2.60
8	250 ng/mL dilution – 3C	2.73
9	250 ng/mL dilution – 3C	2.76
Mean		2.67
SD		0.09
SE		0.03
% CV		3.32

Number of Replicates	Description	mg/mL
Dose Solution IV – Group IV		
1	250 ng/mL dilution – 3A	2.49
2	250 ng/mL dilution – 3A	2.47
3	250 ng/mL dilution – 3A	2.32
4	250 ng/mL dilution – 3B	2.51
5	250 ng/mL dilution – 3B	2.51
6	250 ng/mL dilution – 3B	2.43
7	250 ng/mL dilution – 3C	2.63
8	250 ng/mL dilution – 3C	2.62
9	250 ng/mL dilution – 3C	2.56
Mean		2.50
SD		0.09
SE		0.03
% CV		3.74
Dose Solution V – Group V		
1	250 ng/mL dilution – 3A	2.28
2	250 ng/mL dilution – 3A	2.19
3	250 ng/mL dilution – 3A	2.11
4	250 ng/mL dilution – 3B	2.18
5	250 ng/mL dilution – 3B	1.78
6	250 ng/mL dilution – 3B	2.07
7	250 ng/mL dilution – 3C	1.85
8	250 ng/mL dilution – 3C	2.20
9	250 ng/mL dilution – 3C	2.21
Mean		2.10
SD		0.17
SE		0.06
% CV		8.13
Dose Solutions I-V		
Mean		2.41
SD		0.34
SE		0.11
% CV		14.19

APPENDIX B

Plasma Concentration Data

Males

Animal Number	$\mu\text{g/mL}$
Group I	
101	6.01
102	6.20
103	8.31
104	6.38
105	6.64
106	7.22
107	8.45
108	7.19
109	8.11
110	8.74
Mean	7.32
SD	1.01
SE	0.32
% CV	13.86
Group II	
201	33.40
202	39.43
203	46.42
204	41.89
205	38.72
206	37.35
207	42.64
208	35.11
209	40.96
210	36.45
Mean	39.24
SD	3.89
SE	1.23
% CV	9.90
Group III	
301	38.14
302	39.95
303	42.00
304	45.27
305	41.38
306	43.46
307	48.23
308	50.44
309	40.99
310	42.01
Mean	43.19
SD	3.79
SE	1.20
% CV	8.77

Males (continued)

Animal Number	$\mu\text{g/mL}$
Group IV	
401	30.49
402	31.28
403	40.22
404	41.41
405	36.76
406	35.14
407	38.70
408	35.96
409	38.36
410	42.93
Mean	37.12
SD	4.07
SE	1.29
% CV	10.97
Group V	
501	32.66
502	37.93
503	46.78
504	27.47
505	38.05
506	39.94
507	41.65
508	38.03
509	39.16
510	43.85
Mean	38.55
SD	5.44
SE	1.72
% CV	14.10

Females

Animal Number	$\mu\text{g/mL}$
---------------	------------------

Group I

111	3.05
112	2.41
113	2.52
114	2.27
115	2.87
116	3.33
117	1.67
118	2.13
119	3.90
120	2.67

Mean	2.68
SD	0.64
SE	0.20
% CV	23.77

Group II

211	0.98
212	1.18
213	0.81
214	1.79
215	1.65
216	0.76
217	1.47
218	0.69
219	0.43
220	1.52

Mean	1.13
SD	0.46
SE	0.15
% CV	41.13

Group III

311	1.39
312	0.94
313	0.89
314	1.92
315	0.66
316	1.39
317	1.06
318	0.84
319	0.56
320	2.12

Mean	1.18
SD	0.52
SE	0.17
% CV	44.43

Females (continued)

Animal Number	$\mu\text{g/mL}$
Group IV	
411	0.66
412	0.70
413	0.21
414	0.51
415	0.75
416	0.43
417	1.09
418	LOQ
419	LOQ
420	0.23
Mean	0.57
SD	0.29
SE	0.09
% CV	51.00
Group V	
511*	2.66
512	0.87
513	0.63
514	0.95
515	1.36
516	0.67
517	0.64
518	0.89
519	0.50
520	LOQ
Mean	0.81
SD	0.27
SE	0.09
% CV	33.17

* According to Chauvenet's criterion,⁽⁷⁾ the value of PFOA plasma concentration obtained from rat 511 was not included for the calculation of the mean and the SD.