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BIOANALYTICAL REPORT

Title:	Quantitative Determination of Ammonium Perfluorooctanoate (APFO), Measured as the Perfluorooctanoate Anion (PFOA), in Human Serum from Study DW04 Using Turbo Ion Spray LC/MS/MS
Date:	30 September 2003
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Advion Internal Study ID:	DW04
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Summary and Conclusions

The concentration of ammonium perfluorooctanoate (APFO), measured as the perfluorooctanoate anion (PFOA), was determined in human serum samples collected during the Study DW04 using a selective, accurate, and reproducible analytical method developed by Advion BioSciences, Inc., Ithaca, NY.

Human serum samples (0.025 mL) were extracted by a liquid-liquid extraction procedure to isolate PFOA and the internal standard (9H-hexadecafluorononanoic acid, 9H-HDFNA). Following reconstitution, sample extracts were analyzed by turbo ion spray liquid chromatography/tandem mass spectrometry (LC/MS/MS) in the negative ion mode.

All samples were successfully analyzed within one run. The lower limit of quantitation was 0.05 µg/mL for PFOA. The precision of this assay (CV) as determined from the analysis of quality control (QC) samples for PFOA was ≤4.11%.

ADVION BIOSCIENCES

SIGNATURE PAGE

Title: Quantitative Determination of Ammonium Perfluorooctanoate (APFO), Measured as the Perfluorooctanoate Anion (PFOA), in Human Serum from Study DW04 Using Turbo Ion Spray LC/MS/MS

Advion Report No.: 02331ADJA_DU.DOC

Reported by:

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30 SEP 2003
Date

Authorized for Release by:

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Director of Bioanalysis

30.SEP.03
Date

QAU STATEMENT

Periodic inspections of the bioanalytical portion of Study DW04 were conducted by the Quality Assurance Unit of Advion BioSciences (Advion).

The study was inspected on the following dates:

Phase of Study Inspected	Date of Inspection	Date Reported to Management
Sample Login Binder	8 January 2003	8 January 2003
Data Review	31 December 2002	31 December 2002
Report	17 September 2003	18 September 2003

Based on the inspections and the data reviewed, this report is a complete and accurate representation of the data.

George C. Amedro
George C. Amedro, B.S.
Quality Assurance Auditor

30 Sep 03
Date

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1. INTRODUCTION

1.1. Study Description and Objective

The objective of this study was to determine the concentrations of ammonium perfluorooctanoate (APFO), measured as the perfluorooctanoate anion (PFOA), in human serum samples collected during Study DW04. The study ID was generated by Advion BioSciences, Inc. (Advion) for internal tracking purposes. Human serum samples were analyzed using a selective liquid-liquid extraction, turbo ion spray liquid chromatography/ tandem mass spectrometry (LC/MS/MS) assay. The work described in this bioanalytical report was conducted in accordance with Advion standard operating procedures (SOP).

2. METHODS

2.1. Analytical Procedure(s)

PFOA concentrations were determined in human serum according to the validated LC/MS/MS method (1). Serum samples (0.025 mL) were extracted by a liquid-liquid extraction procedure to isolate the analyte and internal standard (IS) from human serum. Following reconstitution, sample extracts were separated by reversed-phase chromatography on a 2 x 50 mm (5 µm) Betasil C_{18} column (ThermoHypersil-Keystone, Bellefonte, PA) with an initial mobile phase of 45% Eluent B (90:10 methanol: 2 mM ammonium acetate) and 55% Eluent A (10:90 methanol:2 mM ammonium acetate). 9H-hexadecafluorononanoic acid (9H-HDFNA) was used as the IS for PFOA. PFOA concentrations were determined by turbo ion spray liquid chromatography/tandem mass spectrometry (LC/MS/MS) in the negative ion mode.

Following background subtraction (for calibration standards, QC samples, and zero samples), study sample concentrations were determined from a weighted ($1/x^2$), quadratic regression of peak area ratios (peak area of PFOA/peak area of 9H-HDFNA) versus nominal concentration of nine calibration standards. The nominal concentrations of the calibration standards were 0.05, 0.1, 0.25, 0.5, 1, 2, 5, 7.5 and 10 µg/mL PFOA.

The lower limit of quantitation (LLQ) for this assay was 0.05 µg/mL PFOA. Quality control (QC) serum samples at three different concentrations (0.15, 3, and 8 µg/mL PFOA) were analyzed in replicates of three.

The acceptance criteria for calibration standards stipulated that at least three-fourths of the calibration standards must remain on the calibration curve for each analytical run. Calibration standards may be rejected if they show deviations >15% from their nominal concentration (20% at the LLQ). The coefficient of determination (r^2) must be ≥0.98.

The acceptance criteria for the quality control samples stipulated that two-thirds of the individual QC samples must be within ±15% of the nominal concentration, and at least

one-half of the replicates at each QC concentration must be within ±15% of the nominal concentration.

A summary of the sample and assay information for Study DW04 is given in Table 1. Trip blanks were prepared at Advion in advance of the collection of serum samples in this study. The trip blanks were prepared by adding control human serum to collection tubes. The purpose of the trip blanks was as a precautionary means to monitor for potential contamination of study samples during shipment.

2.2. Assay Site

All samples were analyzed at Advion BioSciences, Inc., Ithaca, NY.

2.3. Data Processing

The data were collected using turbo ion spray LC/MS/MS in the negative ion mode. Peak areas were integrated by the Applied BioSystems program MacQuan, version 1.6, residing on a Macintosh computer. Following peak area integration, the results tables from MacQuan were saved as text files, which were used to perform background subtraction, using Excel 98, to correct for the presence of PFOA in the control serum lots. Background-subtracted data were saved as MacQuan text files and uploaded to the Advion file server where a weighted ($1/x^2$) quadratic regression was performed using the software package Watson (v 6.1.0.03, InnaPhase Corp., Philadelphia, PA). Concentration data were downloaded from Watson with three significant figures. Statistics were downloaded from Watson with three decimal places and rounded to three significant figures prior to reporting.

3. RESULTS

3.1. Assay Performance

The performance of the assay for PFOA as determined from the analysis of QC samples is documented in Table 2. The intra-assay precision (CV) of QC samples ranged from 3.14 to 4.11% for PFOA. There was no marked inaccuracy in the results from these QC samples; mean accuracy (RE) ranged from -10.7 to -6.33%.

The performance of daily calibration curves is documented in Table 3. There was no marked inaccuracy in the results from these standards; mean accuracy ranged from -2.60 to 5.00%.

The slope, y-intercept, and coefficient of determination (r^2) for the analytical run are presented in Table 4. The r^2 value was 0.9958 for PFOA in human serum.

All Advion personnel assigned to analyze samples for this project were required to successfully extract QC samples and calibration standards. Results of the extraction were reviewed by a trained analyst against the pre-defined acceptance criteria for this assay. Results are maintained in the study records of Advion.

Representative chromatograms from a PFOA control blank and zero sample are presented in Figures 1 and 2, respectively. Representative chromatograms from PFOA standards 1 and 9 are presented in Figures 3 and 4, respectively.

3.2. Analytical Results for Study DW04

PFOA concentrations in human serum from Study DW04 are presented in Table 5. All data were rounded to no less than three significant figures before reporting in Table 5. The two trip blanks are identified as subject numbers 98009 and 98010.

4. DATA RETRIEVAL

The calculated concentration data from the analysis of human serum samples for Study DW04 are maintained on file in the archives of Advion BioSciences, Inc., Ithaca, NY in Advion Notebook 02331.

5. REFERENCES

1. Advion BioSciences Validation Report 02120VDJA_DU.DOC. Method Validation for the Quantitation of Ammonium Perfluorooctanoate (APFO), Measured as the Perfluorooctanoate Anion (PFOA, 0.05 to 10 µg/mL Concentration Range), in Human Serum by Turbo Ion Spray LC/MS/MS. 12 July 2002.

6. TABLES

Table 1: Summary of Sample and Assay Information for Study DW04

Species/Matrix: Human/Serum

Sample Collection and Storage Information:

Anticoagulant/Stabilizer:	Not applicable
Date Received at Advion:	10 October 2002
Storage Temperature at Advion:	-20 °C
Number of Study Samples Received:	62
Number of Study Samples Analyzed:	62

Assay Information:

Assay Period:	29 October 2002

Analyte:

Analytical Standard:	**PFOA** PFOA (supplied as ammonium perfluorooctanoate, APFO)
Lot No:	421207/1
Source:	Sigma-Aldrich
Internal Standard:	9H-HDFNA
Lot No:	None available
Source:	Sigma-Aldrich

Calibration Range:

Regression Method:	Quadratic
Weighting Factor:	$1/x^2$
Lower Limit of Quantitation:	0.05 µg/mL
Upper Limit of Quantitation:	10 µg/mL

Table 3: Precision and Accuracy for PFOA Calibration Standards in Human Serum for Study DW04

	PFOA Concentration (µg/mL)								
Run Number	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9
	0.05	0.1	0.25	0.5	1	2	5	7.5	10
1	0.0444	0.0984	0.232	0.495	0.948	1.93	4.92	7.68	10.2
	0.0534	0.111	0.262	0.512	1.00	2.11	4.85	7.53	9.86
Mean	0.0489	0.105	0.247	0.504	0.974	2.02	4.89	7.61	10.0
CV (%)	NA	NA	NA	NA	NA	NA	NA	NA	NA
RE (%)	-2.20	5.00	-1.20	0.800	-2.60	1.00	-2.20	1.47	0.00

CV = (SD/Mean)*100

RE = [(Mean-Nominal)/Nominal]*100

NA = Not applicable

Table 4: **Calibration Curve Statistics for the Determination of PFOA in Human Serum for Study DW04**

Run Number	Quadratic Coefficient (A)	Linear Coefficient (B)	Intercept (C)	Coefficient of Determination (r^2)
1	-0.004962	0.357472	-0.001110	0.9958

$y = Ax^2 + Bx + C$, weighted $1/x^2$, after background subtraction

Table 5: **Concentrations of PFOA in Human Serum Samples from Study DW04**

Subject Number	PFOA Concentration (µg/mL)
57178	0.168
57179	0.175
57180	0.0788
57181	1.65
57182	<LLQ
57183	<LLQ
57184	<LLQ
57185	0.0561
57186	<LLQ
57187	1.13
57188	1.11
57189	<LLQ
57190	<LLQ
57191	<LLQ
57192	<LLQ
57193	<LLQ
57194	<LLQ
57195	<LLQ
57196	0.0543
57197	<LLQ
57198	0.0978
57199	0.347
57200	0.127
57201	0.0834
57202	0.277
57203	0.0506
57204	0.297
57205	0.233
57206	0.0527
57207	0.174
57208	0.125
57209	<LLQ
57210	0.0565
57211	0.210
57212	<LLQ
57213	<LLQ
57214	0.658

...continued

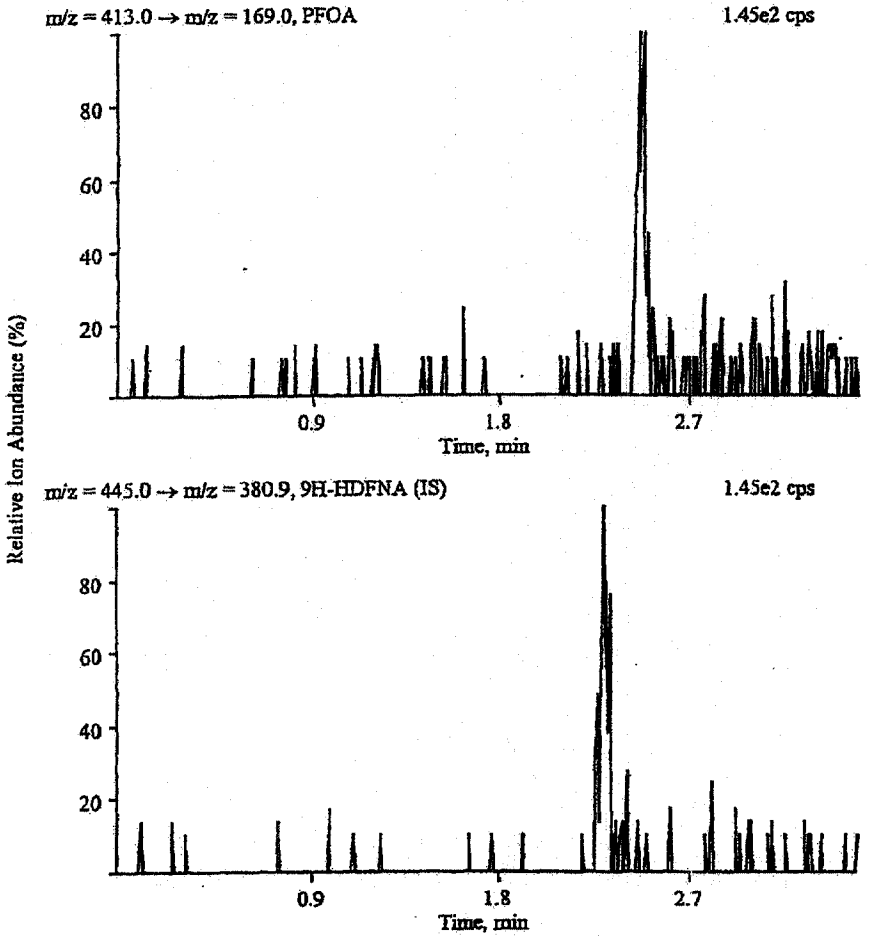
Table 5: (Continued) Concentrations of PFOA in Human Serum Samples from Study DW04

Subject Number	PFOA Concentration (µg/mL)
57215	0.0910
57216	0.0750
98009	<LLQ
98010	<LLQ
98039	<LLQ
98040	<LLQ
98041	0.244
98047	0.0598
98048	0.137
98049	<LLQ
98055	0.106
98056	0.199
98133	<LLQ
98134	0.0885
98135	0.242
98136	0.226
98137	0.359
98138	0.122
98139	0.0629
98140	<LLQ
98141	0.127
98142	0.264
98143	0.0807
98144	<LLQ
98145	<LLQ
98146	0.0576
98147	0.313

<LLQ: Less than the Lower Limit of Quantitation (0.05 µg/mL)

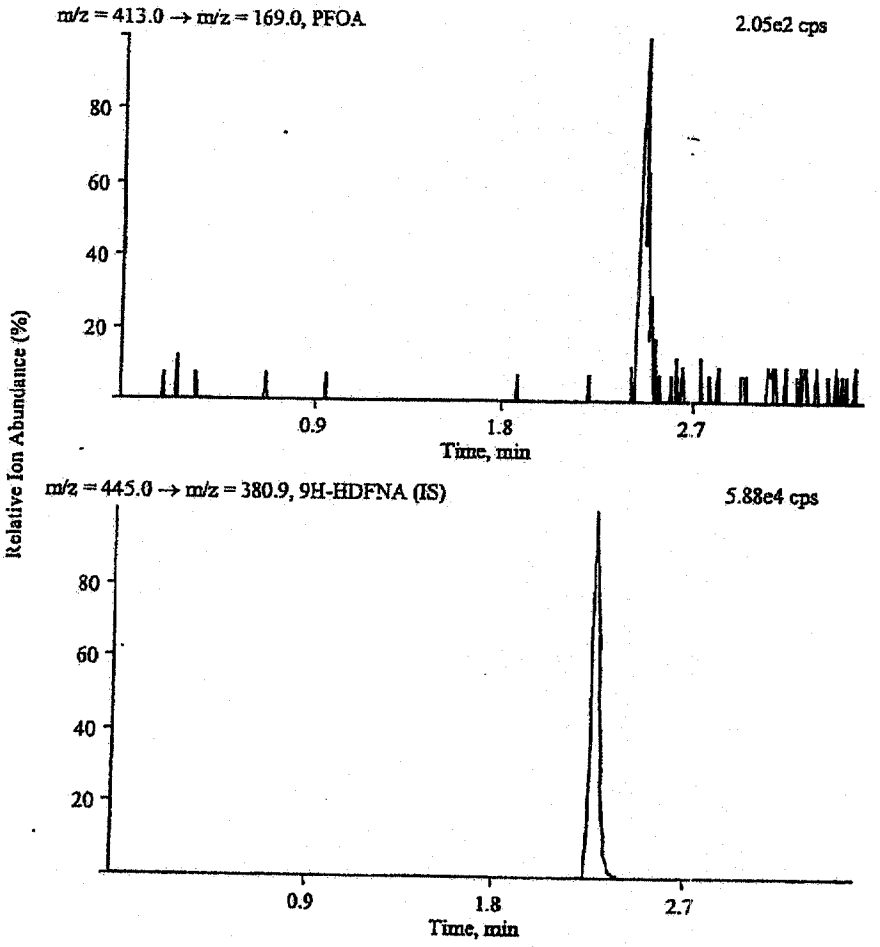
7. FIGURES

Figure 1: Selected Reaction Monitoring Chromatograms of PFOA and the Internal Standard in Control Blank Human Serum Extract



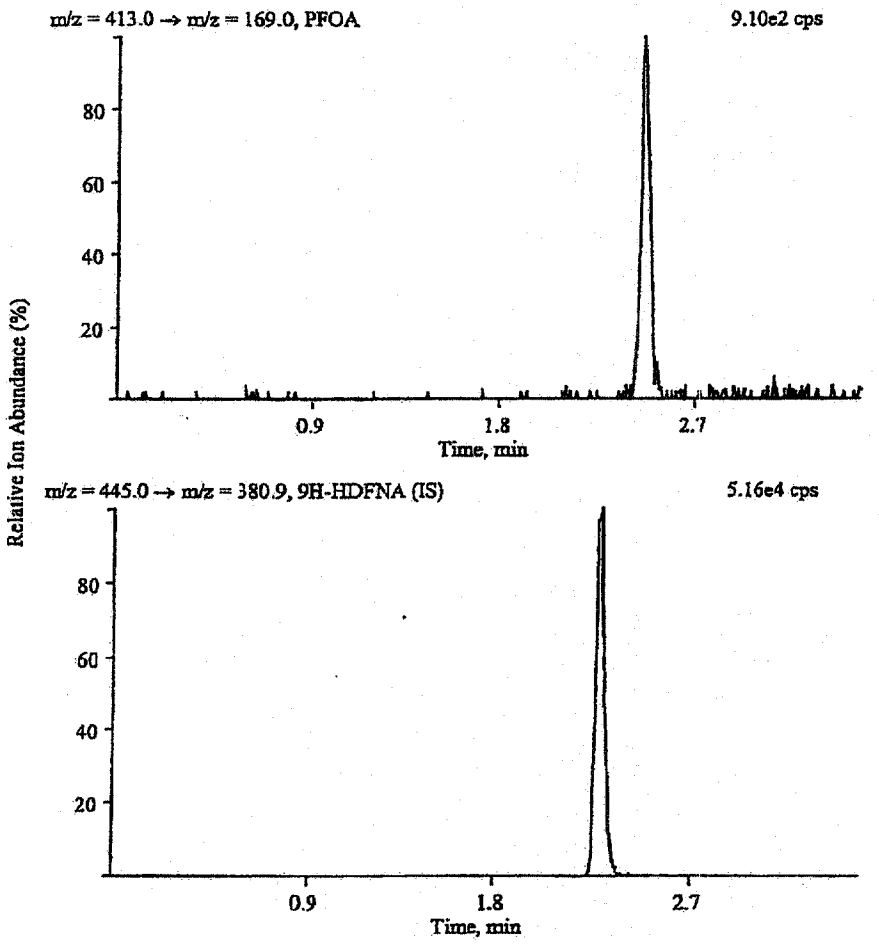
Control Blank, Run 1, Injection 7

Figure 2: **Selected Reaction Monitoring Chromatograms of PFOA and the Internal Standard from a Human Serum Extract Containing Internal Standard Only (Zero Sample)**



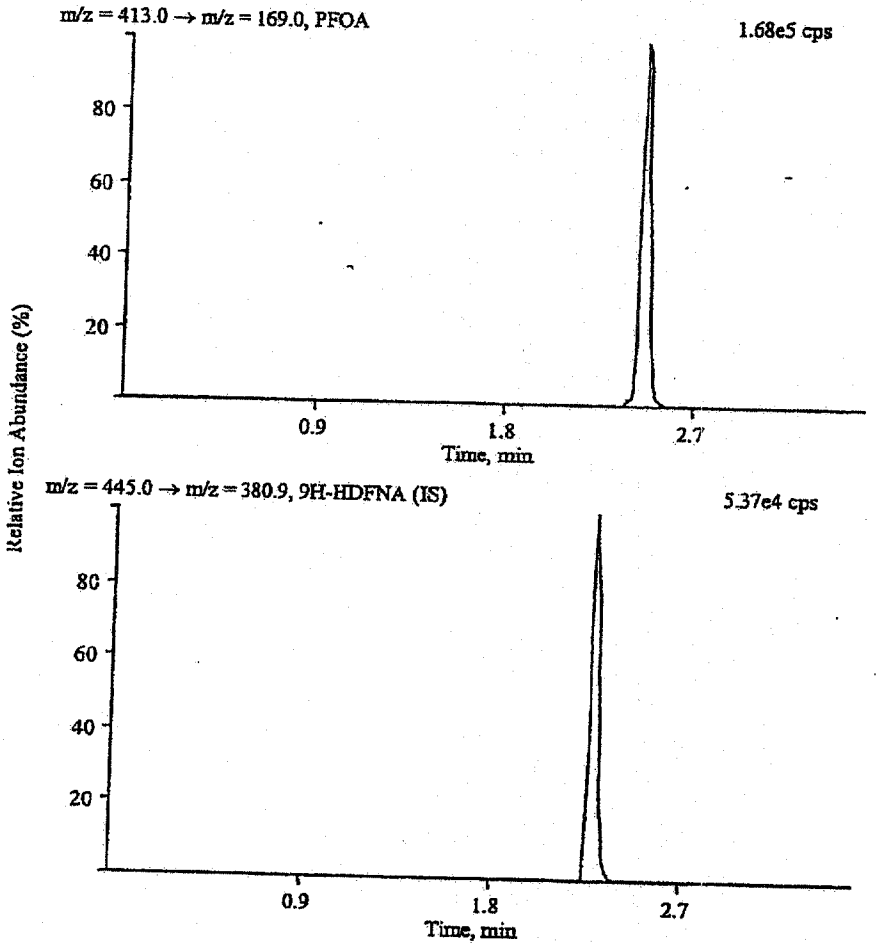
Zero Sample, Run 1, Injection 93

Figure 3: **Selected Reaction Monitoring Chromatograms of PFOA and the Internal Standard from a Standard 1 Human Serum Extract (0.05 µg/mL)**



Standard 1, Run 1, Injection 8

Figure 4: **Selected Reaction Monitoring Chromatograms of PFOA and the Internal Standard from a Standard 9 Human Serum Extract (10 µg/mL)**



Standard 9, Run 1, Injection 16