AR 226-1354

Protein Binding of Perfluorohexane Sulfonate, Perfluorooctane Sulfonate and Perfluorooctanoate to Plasma (Human, Rat, and Monkey), and Various Human-Derived Plasma Protein Fractions

STUDY ID: 9921.7

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SUMMARY

The percent protein binding of four perfluoronated compounds in seven different human-derived plasma protein fractions was determined. Additionally, protein binding for the same four compounds was measured in rat, monkey and human plasma at five different concentrations. Each test article was incubated for 15 minutes at 37 °C in each plasma protein fraction or plasma sample. The protein bound test article was separated from the non-bound test article by ultrafiltration using Centrifree® filtration devices with a nominal molecular weight (NMW) cutoff of 30,000 daltons. The eluent from the ultrafiltration was analyzed for test article concentration relative to a solvent standard.

KEY PERSONNEL

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1. OBJECTIVE

The objective of this study was to determine the percent of protein binding for each test article in human, rat, and monkey plasma as well as various human-derived plasma protein fractions.

2. SAFETY

All necessary procedures to ensure the safety of the analysts were based on information contained in the Material Safety and Data Sheets (MSDS), provided by the sponsor for the test articles used in this study. Procedures outlined in SOP 2-29-3 for handling human and primate plasma as well as the human-derived plasma protein fractions were also followed.

3. COMPLIANCE

This work was performed using various previously validated analytical methods (3548, 3606, 3607). While this work was not audited in compliance with GLP regulations, it was performed in the spirit of the regulations using calibrated and validated instrumentation.

4. EXPERIMENTAL

4.1 Analytical Procedures

Individual aqueous solutions of each human derived plasma protein fraction were prepared at two concentrations as indicated in the table below:

Plasma Protein Fraction	Physiological Plasma Protein Concentration(1)	Prepared Plasma Protein Concentration (~10 % Physiological)	Prepared Plasma Protein Concentration (100% Physiological)
Albumin	3500-5500 mg/100 mL	443 mg/100 mL	4600 mg/100 mL
Gamma-Globulin	965-2515 mg/100 mL	196 mg/100 mL	1768 mg/100 mL
Alpha-Globulin	507-1037 mg/100 mL	136 mg/100 mL	1212 mg/100 mL
Fibrinogen	200-600 mg/100 mL	38 mg/100 mL	408 mg/100 mL
Alpha-2-Macroglobulin	150-420 mg/100 mL	67 mg/100 mL	200 mg/100 mL
Transferrin	200-320 mg/100 mL	42 mg/100 mL	297 mg/100 mL
Beta-Lipoproteins	280-440 mg/100 mL	20 mg/100 mL	200 mg/100 mL

Stock solutions of each test article were separately spiked into the plasma protein fractions or a control plasma matrix to produce a final concentration of 10 µg/mL. The resulting mixture was vortexed for 1 minute and incubated at 37 °C for 15 minutes. Upon completion of the incubation, each sample was again vortexed for 1 minute and placed into a Centrifree ultrafiltration device (NMW cutoff = 30,000 daltons) and centrifuged at 2000 x g for 30 minutes. The ultrafiltrate was analyzed to determine the amount of unbound test article.

4.2 Results

The results of all of the analyses are presented as percent bound test article in Table I-III at the end of the report.

5.0 Conclusion

The protein binding of four perfluoronated compounds was evaluated in seven separate human-derived plasma protein fractions at two different protein fraction concentrations (Table I and II). Additionally, protein binding for the same four compounds was evaluated in rat, human, and monkey plasma at 5 different concentrations (Table III). The data in Table I and II shows that albumin is by far the largest single protein binder for three of the four compounds tested at both concentrations. The fourth compound, PFOS, was found to be highly bound by both albumin and beta-lipoproteins. The protein binding experiments for the rat, human, and monkey plasma show very high levels of binding at all concentrations tested and no evidence of saturation.

Table I
Percent Binding to Human Plasma Protein Fractions (~10% Physiological Conc.)

	Albumin	Gamma-Globulin	Alpha-Globulin	Fibrinogen	Alpha-2-Macroglobulin	Transferrin	Beta-Lipoproteins
PFHS	>99.5	15.0	33.6	15.6	9.0	8.0	25.8
PFOS	99.0	6.3	49.9	<0.1	12.5	7.2	90.1
PFOC	96.4	3.5	28.5	5.4	7.9	1.0	19.6

Table II
Percent Binding to Human Plasma Protein Fractions (100% Physiological Conc)

	Albumin	Gamma-Globulin	Alpha-Globulin	Fibrinogen	Alpha-2-Macroglobulin	Transferrin	Beta-Lipoproteins
PFHS	>99.9	26.1	13.7	<0.1	<0.1	6.4	64.1
PFOS	99.8	24.1	59.4	<0.1	<0.1	<0.1	95.6
PFOC	99.7	3.0	11.0	<0.1	<0.1	2.1	39.6

Table III
% Protein Binding to Rat, Human and Monkey Plasma

Nominal Concentration (ppm)				Perfluorohexanesulfonate			Perfluorooctanesulfonate			Perfluorooctanecarboxalate		
				Rat	Monkey	Human	Rat	Monkey	Human	Rat	Monkey	Human
1				~100	~100	~100	~100	~100	99.4	~100	~100	~100
10				~100	~100	~100	99.8	99.9	99.9	99.5	99.8	99.9
100				99.9	99.9	100	99.7	99.9	99.9	98.6	99.8	99.9
250				99.1	99.8	99.9	99.5	99.9	99.9	97.6	99.8	99.6
500				98.2	99.5	99.4	99.0	99.9	99.9	97.3	99.5	99.4

% Binding Values reported as "~100" reflect a nonquantifiable amount of test article in the plasma water BQL < 6.25 ng/mL

6.0 References

(1). Koplar, L., Pesce, A., "Clinical Chemistry (Theory, Analysis and Correlation) 1984, pg. 1416, C.V. Mosby Publishing Co.

7.0 Review and Approvals

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06-03-03
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ANALYTICAL METHOD

Method No.: BACG-3548

Title: Determination of Perfluorooctanecarboxalate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

1.0 PRINCIPLE

Serum or urine samples are obtained from cynomolgus monkeys treated with Perfluorooctanecarboxalate (PFOC). The serum or urine (e.g., 0.5 mL) containing (PFOC) is fortified with an internal standard (IS), perfluorohexanesulfonate (PFHS). The samples are then mixed with an ion-pairing reagent, buffer and water, followed by extraction with ethyl acetate. The ethyl acetate layer is removed, evaporated to dryness, reconstituted in 95% methanol containing 1.5 % formic acid, 5 % 5mM ammonium acetate, filtered, and transferred to autosampler vials, and analyzed by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS). The range of reliable results extends from about 20 to 100,000 ng/mL of PFOC in serum and from 10 to 500 ng/mL in urine. Samples containing PFOC at concentrations greater than 100,000 ng/mL may be diluted with control blank matrix so that the concentration of PFOC will be within the range of reliable results prior to analysis.

The mass spectrometry of PFOC and PFHS is accomplished in the negative ion mode. The ion spray source voltage is set at - 2000 volts which is low enough to greatly reduce the formation of other potentially interfering ions extracted from the matrix. In order to maximize sensitivity, this method is based on mixed reaction monitoring of the negative fragment ion $(M\text{-}COOH)^-$ for PFOC.

CAUTION: Since primates may carry a number of zoonoses, all unpreserved tissues, including blood, plasma and serum, are to be considered as biohazards and handled with **universal precautions.** Refer to SOP number SRI 2-5-5 for a description of safety procedures to be used when handling unpreserved primate tissue.

2.0 REAGENTS AND SOLUTIONS

The listed reagents or their equivalents may be used.

2.1 Neat Reagents

ANALYTICAL METHOD

Method No.: BACG-3548

Title: Determination of Perfluorooctanecarboxalate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

2.1.1 Water, deionized and organic free (from in-house purification system; e.g., Ingalls 210N)

2.1.2 Methanol, HPLC grade

2.1.3 Perfluorooctanecarboxalate (analyte), as provided by the client

2.1.4 Perfluorohexanesulfonate (internal standard), 97%

2.1.5 Ammonium acetate, HPLC grade

2.1.6 Blank control monkey serum

2.1.7 Sodium Carbonate, Certified ACS Grade or equivalent

2.1.8 Sodium Bicarbonate, Certified ACS Grade or equivalent

2.1.9 Ethyl Acetate, HPLC grade

2.1.10 Tetrabutylammonium Hydrogen Sulfate

2.1.11 Sodium Hydroxide 50% solution, Certified grade or equivalent

2.1.12 Blank control monkey urine

2.1.13 Formic Acid

2.2 **Prepared Solutions**

Appropriate changes in the solutions may be made at the discretion of the analyst

2.2.1 5 mM Ammonium acetate in organic free water

2.2.1.1 For example, to prepare 4 liters, measure out ammonium acetate (e.g., 1.542 g) and add in organic-free water (e.g., 4 L). Mix well and filter through HPLC mobile phase filtration apparatus.

2.2.2 TBA Ion-Pairing Solution (0.5 M tetrabutylammonium hydroxide)

2.2.2.1 For example, to prepare 25 mL, dissolve 4.24 g of tetrabutylammonium hydrogen sulfate in deionized water and adjust the pH to 10 with 50% NaOH solution. **Note: a more dilute solution of NaOH in water may be used to effect smaller pH adjustments.**

2.2.3 Carbonate/Bicarbonate Buffer Solution

2.2.3.1 For example, to prepare 100 mL, dissolve approximately 2.65 g of sodium carbonate and approximately 2.10 g of sodium bicarbonate in 100 mL of deionized water. Mix well to ensure complete dissolution.

3.0 INSTRUMENTS, MATERIALS, AND APPARATUS

The following or their equivalents may be used.

3.1 HPLC pump(s), autosampler, and triple quadrupole mass spectrometer

3.2 Autosampler vials with inserts

3.3 Vortex mixers (e.g., touch mixer and IKA-Vibrax ® platform mixer)

3.4 Solvent-concentration apparatus (e.g., Zymark Turbo-Vap ® with source of nitrogen)

3.5 HPLC mobile phase filtration apparatus

3.6 Filters for HPLC mobile phase filtration apparatus (e.g., Nylon-66, 0.20 μm)

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3.7 Analytical balance

3.8 Volumetric flasks (e.g., 10 and 25 mL)

3.9 Disposable Pasteur pipets

3.10 Micropipettor(s) with tips

3.11 Culture tubes with teflon-lined caps

3.12 Centrifuge

3.13 Assorted glassware and syringes

3.14 Culture tubes (vials) for use with solvent-concentration apparatus

3.15 1 mL Plastic syringes with 0.2 μm PVDF syringe filters

3.16 Variable speed horizontal platform shaker

3.17 pH meter

4.0 PREPARATION OF STOCKS AND WORKING STOCKS

Appropriate changes in the concentrations of the solutions may be made at the discretion of the analyst. Actual dilutions will be documented on the preparation sheets.

4.1 Main Stock Solution of PFOC ~1000 µg/mL

4.1.1 Prepare an ~1000 µg/mL solution of PFOC in deionized organic-free water (e.g., accurately weigh about 10 mg PFOC into a 10-mL volumetric flask). Add deionized organic-free water to dissolve. Dilute to the mark. Alternatively, weigh the compound

into an appropriate vessel (e.g., culture tube) and add 10 mL of deionized organic-free water. Mix well. Transfer the solution to a clean vessel if desired.

4.2 **Stock Solution of Internal Standard (PFHS), ~200 µg/mL**

4.2.1 Prepare an ~200 µg/mL solution of PFHS in deionized organic-free water (e.g., accurately weigh about 10 mg into a 50-mL volumetric flask). Add deionized organic-free water to dissolve and dilute to the mark with deionized organic-free water. Alternatively, weigh the compound into a an appropriate vessel (e.g., culture tube) and add 50 mL of deionized organic-free water. Mix well. Transfer the solution to a clean vessel if desired.

4.3 **Spiking solution of Internal Standard (PFHS), ~ 50µg/mL**

4.3.1 Prepare an ~ 50 µg/mL solution of PFHS in deionized organic- free water by pipetting 2 mL of the 200 µg/mL solution into a culture tube and add 6 mL of deionized water. Mix well.

4.4 **Working Stock Solutions of PFOC**

4.4.1 To prepare working stock solutions, make the proper dilutions as shown in the following table. Prepare in 10-mL volumetric flasks or other appropriate glassware. If desired a modified dilution scheme can be used and documented in the study records.

Working Stock Level (WSL) Approximate Concentration (ng/mL)	Volume of PFOC solution	Final Volume in deionized organic free water (mL)
500,000	5 mL of 1000 µg/mL	10
250,000	5 mL of 500,000 ng/mL	10

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62,500	2.5 mL of 250,000 ng/mL	10
31,250	5 mL of 62,500 ng/mL	10
15,625	5 mL of 31,250 ng/ mL	10
5,000	50 µL of 1000 µg/mL stock	10
2,500	5 mL of 5000 ng/mL stock	10
1,000	4 mL of 2500 ng/mL stock	10
500	5 mL of 1000 ng/mL stock	10
250	5 mL of 500 ng/mL stock	10

4.4.2 Summary of concentrations of serum standards:

Standard Level	Volume and Spike Conc.	Approximate Concentration of PFOC in serum (ng/mL)
A	50 µL of 1000 µg/mL	100,000
B	37.5 µL of 1000 µg/mL	75,000
C	25 µL of 1000 µg/mL	50,000
D	12.5 µL of 1000 µg/mL	25,000
E	10 µL of 1000 µg/mL	20,000

ANALYTICAL METHOD

Method No.: BACG-3548

Title: Determination of Perfluorooctanecarboxalate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

F	10 µL of 500,000 ng/mL	10,000
G	10 µL of 250,000 ng/mL	5,000
H	16 µL of 62,500 ng/mL	2,000
I	16 µL of 31,250 ng/mL	1,000
J	16 µL of 15,625 ng/mL	500
K	20 µL of 5,000 ng/mL	200
L	10 µL of 5,000 ng/mL	100
M	10 µL of 2,500 ng/mL	50
N	10 µL of 1,000 ng/mL	20
O	10 µL of 500 ng/mL	10
P	10 µL of 250 ng/mL	5

5.0 PREPARATION OF SPIKED STANDARDS AND BLANKS

Appropriate changes in the concentrations of the solutions may be made at the discretion of the analyst.

5.1 Multiple (e.g., about three) sets of matrix standards and a matrix blank (blank + IS) are analyzed with each set of unknown samples. A matrix double blank (blank-IS) may also be analyzed if desired.

5.2 Into individual ~20-mL culture tubes, pipet blank matrix (e.g., 0.5 mL). Pipet in the appropriate volume as described in the table above for each standard. For the blanks, pipet 10 µL of organic-free water instead of the working stock solution. Add the 10 µL

ANALYTICAL METHOD

Method No.: BACG-3548

Title: Determination of Perfluorooctanecarboxalate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

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of internal standard stock (~50 µg/mL) to each tube except the blank-IS (pipet 10 µL of organic-free water instead) and vortex for ~5 seconds.

5.3 To each tube add 500 µL of the TBA ion-pairing solution, 1 mL of carbonate/bicarbonate buffer, and 1 mL of deionized organic free water. Vortex each tube for about 5 seconds.

5.4 Add 2.5 mL of ethyl acetate and extract on horizontal mixer for 1 hour at a low speed setting.

5.5 Remove the tube from the shaker and place in a centrifuge (e.g., 2500 rpm) for about 5 minutes.

5.6 Take off the top ethyl acetate layer and put it into a clean tube and evaporate to dryness (e.g., ~ 50 minutes in the Turbo-Vap ®) with a gentle stream of nitrogen and moderate heat (e.g., 50 °C).

5.7 Reconstitute the residue in 500 µL of 5% 5 mM ammonium acetate: 95% methanol containing 1.5% formic acid and vortex briefly to mix. Filter the samples through 0.2 µm PVDF or Nylon syringe filters into autosampler vials.

6.0 PREPARATION OF SAMPLES

6.1 Allow each serum sample to thaw to room temperature. Vortex each sample briefly, but vigorously. Pipet an aliquot of each sample (e.g., 0.5 mL) into individual ~20-mL culture tubes. If necessary, dilute an aliquot of any sample with blank matrix so that the expected concentration of the test article being analyzed will fall within the concentration range of the standard curve. Add 10 µL of internal standard stock (~ 50 µg/mL) to each tube and vortex for a couple of seconds.

6.2 To each tube add 500 µL of the TBA ion-pairing solution, 1 mL of carbonate/bicarbonate buffer, and 1 mL of deionized organic free water. Vortex each tube for about 5 seconds.

6.3 Add 2.5 mL of ethyl acetate and extract on horizontal mixer for 1 hour at a low speed setting.

6.4 Remove the tube from the shaker and place in a centrifuge (e.g., 2500 rpm) for about 5 minutes.

6.5 Take off the top ethyl acetate layer and put it into a clean tube and evaporate to dryness (e.g., ~ 50 minutes in the Turbo-Vap ®) with a gentle stream of nitrogen and moderate heat (e.g., 50 °C).

6.6 Reconstitute the residue in 500 µL of 5% 5 mM ammonium acetate: 95% methanol containing 1.5% formic acid and vortex briefly to mix. Filter the samples through 0.2 µm PVDF or Nylon syringe filters into autosampler vials. Cap vials for analysis.

7.0 ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY/MASS SPECTROMETRY (HPLC/MS/MS)

7.1 Conditions are to be optimized if necessary.

7.1.1 HPLC Conditions

Analytical Column:	Keystone Scientific Aquasil C18, 150 mm x 2 mm ID, or equivalent
Guard Column:	Keystone Aquasil C18 10 mm x 2 mm
Elution Flow rate:	400 µL/min.
Injection volume:	3 µL
Mobile phase:	A: 5mM ammonium acetate buffer B: 1.5% formic acid in methanol
Gradient Profile:	0 - 0.5 min. 50%A : 50%B 0.5 - 7.5 min. 10% A : 90% B linear gradient 7.5-8 min. 10%A : 90% B step gradient

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8-11 min. 50%A : 50%B

Temperature: Ambient

7.1.2 PE Sciex API 3000 Triple Quadrupole Mass Spectrometer Conditions
Software: PE Sciex TurboQuan

Turboion Spray Source
Note: Values listed under "MS/MS Acquisition Conditions" override parameters in this table.

Auxiliary Gas: Air (e.g., Grade 0.1) at 85 pounds per square inch

Parameter	Value
IS	-2000
NC	0
TEM	400
OR	-20
RNG	-120
Q0	5
IQ1	6
ST	10
RO1	6
IQ2	10
RO2	30
ST3	40
RO3	32
DF	250
CEM	1800

ANALYTICAL METHOD

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Title: Determination of Perfluorooctanecarboxalate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

Parameter	Value
NEB	15
CUR	6
CAD	5
QPE	0
POL	1
VCM	0
IPE	0

MS/MS Acquisition Conditions

Scan type: MRM
Polarity: Negative
Acquisition mode: Profile
Pause time: 5 milliseconds

Masses requested:

PFOC:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (ms)
412.9	368.9	200

PFHS (IS)

Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (ms)
398.9	398.9	200

Parameter	Start	Stop
RO2	50	50
ST3	60	60
RO3	52	52

ANALYTICAL METHOD

Method No.: BACG-3548

Title: Determination of Perfluorooctanecarboxalate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

8.0 CALCULATIONS

8.1 At the end of the analytical run, review each chromatogram to ensure the retention time, peak shape, and peak height and peak area determination of the test article and the IS are acceptable. The data may be smoothed as appropriate. For quantitation, use the ion profiles at the following mass-to-charge ratios:

Analyte	Ion Profile
PFOC	412.9 to 368.9
PFHS	398.9 to 398.9

8.2 Plot the peak area response of PFOC divided by the peak area response of the IS (PFHS) from all standards versus the concentration of the test article in the standards. Alternatively, the peak heights may be used instead of peak areas. Obtain the best curve fit of the data (e.g., quadratic fit weighted with 1/concentration of the test article or a quadratic fit). Note: The best curve fit may be dependent on the range of the standard curve and it may be necessary to have more than one standard curve for various concentration ranges using the following:

$$y = ax^2 + bx + c$$

where y = Peak height response of PFOC divided by peak height response of the IS (PFHS) in standards.
x = Concentration of the PFOC in standards.
a, b, c = Constants derived from the regression analysis.

8.3 Using the standard curve, calculate the level of PFHC in each unknown sample. Correct the results of samples for any dilutions.

ANALYTICAL METHOD

Method No.: BACG-3548

Title: Determination of Perfluorooctanecarboxalate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

NOTE: Due to unresolvable interferences with the test article and/or internal standard from the matrix, external standard quantitation may be used at the discretion of the supervising mass spectrometrist.

9.0 ACCEPTANCE AND REJECTION CRITERIA

9.1 Refer to SOP SRI 91-3 for acceptance/rejection criteria except acceptable accuracy for standards is 80-120% of theoretical.

10.0 REPORTING

10.1 Results of all analyses are tabulated, and the raw data, original chromatograms, and reports are to be filed in the appropriate study file.

Authors: _____ 12-27-00
Gregory S. Gorman, Ph.D., Staff Chemist
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Date

Approved by: _____ 12-27-00
James D. Johnson MS, MBA, Manager
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Date

ANALYTICAL METHOD

Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

1.0 PRINCIPLE

Serum or urine samples are obtained from cynomolgus monkeys treated with Perfluorohexanesulfonate (PFHS). The serum or urine (e.g., 0.5 mL) containing (PFHS) is fortified with an internal standard (IS), Perfluorooctanecarboxalate (PFOC). The samples are then mixed with an ion-pairing reagent, buffer and water, followed by extraction with ethyl acetate. The ethyl acetate layer is removed, evaporated to dryness, reconstituted in 95% methanol containing 1.5 % formic acid, 5 % 5mM ammonium acetate, filtered, and transferred to autosampler vials, and analyzed by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS). The range of reliable results extends from about 5 to 20,000 ng/mL of PFHS in serum and from 10 to 500 ng/mL in urine. Samples containing PFHS at concentrations greater than 20,000 ng/mL may be diluted with control blank matrix so that the concentration of PFHS will be within the range of reliable results prior to analysis.

The mass spectrometry of PFHS and PFOC is accomplished in the negative ion mode. The ion spray source voltage is set at - 2000 volts which is low enough to greatly reduce the formation of other potentially interfering ions extracted from the matrix.

CAUTION: Since primates may carry a number of zoonoses, all unpreserved tissues, including blood, plasma and serum, are to be considered as biohazards and handled with **universal precautions.** Refer to SOP number SRI 2-5-5 for a description of safety procedures to be used when handling unpreserved primate tissue.

2.0 REAGENTS AND SOLUTIONS

The listed reagents or their equivalents may be used.

2.1 Neat Reagents

2.1.1 Water, deionized and organic free (from in-house purification system; e.g., Ingalls 210N)

2.1.2 Methanol, HPLC grade

ANALYTICAL METHOD

Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

2.1.3 Perfluorohexanesulfonate (analyte), as provided by the client

2.1.4 Perfluorooctanecarboxalate (internal standard), 97%

2.1.5 Ammonium acetate, HPLC grade

2.1.6 Blank control monkey serum

2.1.7 Sodium Carbonate, Certified ACS Grade or equivalent

2.1.8 Sodium Bicarbonate, Certified ACS Grade or equivalent

2.1.9 Ethyl Acetate, HPLC grade

2.1.10 Tetrabutylammonium Hydrogen Sulfate, Aldrich 97%

2.1.11 Sodium Hydroxide 50% solution, Certified grade or equivalent

2.1.12 Blank control monkey urine

2.1.13 Formic Acid, 88%

2.2 **Prepared Solutions**

Appropriate changes in the solutions may be made at the discretion of the analyst

2.2.1 5 mM Ammonium acetate in organic free water

2.2.1.1 For example, to prepare 4 liters, measure out ammonium acetate (e.g., 1.542 g) and add in organic-free water (e.g., 4 L). Mix well and filter through HPLC mobile phase filtration apparatus.

2.2.2 TBA Ion-Pairing Solution (0.5 M tetrabutylammonium hydroxide)

2.2.2.1 For example, to prepare 25 mL, dissolve approximately 4.24 g of tetrabutylammonium hydrogen sulfate in deionized water and adjust the pH to 10 with 50% NaOH solution. **Note: a more dilute solution of NaOH in water may be used to effect smaller pH adjustments.**

2.2.3 Carbonate/Bicarbonate Buffer Solution for Serum (0.25M/0.25M)

2.2.3.1 For example, to prepare 100 mL, dissolve approximately 2.65 g of sodium carbonate and approximately 2.10 g of sodium bicarbonate in 100 mL of deionized water. Mix well to ensure complete dissolution.

2.2.4 Carbonate/Bicarbonate Buffer Solution for Urine (1.0M/1.0M)

2.2.4.1 For example, to prepare 100 mL, dissolve approximately 10.6 g of sodium carbonate and approximately 8.4 g of sodium bicarbonate in 100 mL of deionized water. Mix well to ensure complete dissolution.

3.0 INSTRUMENTS, MATERIALS, AND APPARATUS

The following or their equivalents may be used.

3.1 HPLC pump(s), autosampler, and triple quadrupole mass spectrometer

3.2 Autosampler vials with inserts

3.3 Vortex mixers (e.g., touch mixer and IKA-Vibrax ® platform mixer)

3.4 Solvent-concentration apparatus (e.g., Zymark Turbo-Vap ® with source of nitrogen)

3.5 HPLC mobile phase filtration apparatus

3.6 Filters for HPLC mobile phase filtration apparatus (e.g., Nylon-66, 0.20 µm)

ANALYTICAL METHOD

Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

3.7 Analytical balance

3.8 Volumetric flasks (e.g., 10 and 25 mL)

3.9 Disposable Pasteur pipettes

3.10 Micropipettor(s) with tips

3.11 Culture tubes with teflon-lined caps

3.12 Centrifuge

3.13 Assorted glassware and syringes

3.14 Culture tubes (vials) for use with solvent-concentration apparatus

3.15 1 mL Plastic syringes with 0.2 µm PVDF syringe filters

3.16 Variable speed horizontal platform shaker

3.17 pH meter

4.0 PREPARATION OF STOCKS AND WORKING STOCKS

Appropriate changes in the concentrations of the solutions may be made at the discretion of the analyst. Actual dilutions will be documented on the preparation sheets.

4.1 Main Stock Solution of PFHS ~1000 µg/mL

4.1.1 Prepare an ~1000 µg/mL solution of PFHS in deionized organic-free water (e.g., accurately weigh about 10 mg PFHS into a 10-mL volumetric flask). Add deionized organic-free water to dissolve. Dilute to the mark. Alternatively, weigh the compound

into an appropriate vessel (e.g., culture tube) and add 10 mL of deionized organic-free water. Mix well. Transfer the solution to a clean vessel if desired.

4.2 **Stock Solution of Internal Standard (PFOC), ~200 µg/mL**

4.2.1 Prepare an ~200µg/mL solution of PFOC in deionized organic-free water (e.g., accurately weigh about 10 mg into a 50-mL volumetric flask). Add deionized organic-free water to dissolve and dilute to the mark with deionized organic-free water. Alternatively, weigh the compound into a an appropriate vessel (e.g., culture tube) and add 50 mL of deionized organic-free water. Mix well. Transfer the solution to a clean vessel if desired.

4.3 **Spiking solution of Internal Standard (PFOC), ~ 50µg/mL**

4.3.1 Prepare an ~ 50 µg/mL solution of PFOC in deionized organic- free water by pipetting 2 mL of the 200 µg/mL solution into a culture tube and add 6 mL of deionized water. Mix well.

4.4 **Working Stock Solutions of PFHS**

4.4.1 To prepare working stock solutions, make the proper dilutions as shown in the following table. Prepare in 10-mL volumetric flasks or other appropriate glassware. If desired a modified dilution scheme can be used and documented in the study records.

ANALYTICAL METHOD

Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

Working Stock Level (WSL) Approximate Concentration (ng/mL)	Volume of PFHS solution	Final Volume in deionized organic free water (mL)
500,000	5 mL of 1000 µg/mL	10
250,000	5 mL of 500,000 ng/mL	10
62,500	2.5 mL of 250,000 ng/mL	10
31,250	5 mL of 62,500 ng/mL	10
15,625	5 mL of 31,250 ng/ mL	10
5,000	50 µL of 1000 µg/mL stock	10
2,500	5 mL of 5000 ng/mL stock	10
1,000	4 mL of 2500 ng/mL stock	10
500	5 mL of 1000 ng/mL stock	10
250	5 mL of 500 ng/mL stock	10

ANALYTICAL METHOD

Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

4.4.2 Summary of concentrations of serum standards:

Standard Level	Volume and Spike Conc.	Approximate Concentration of PFHS in serum (ng/mL)
A	20 µL of 500,000 ng/mL	20,000
B	10 µL of 500,000 ng/mL	10,000
C	10 µL of 250,000 ng/mL	5,000
D	16 µL of 62,500 ng/mL	2,000
E	16 µL of 31,250 ng/mL	1,000
F	16 µL of 15,625 ng/mL	500
G	20 µL of 5,000 ng/mL	200
H	10 µL of 5,000 ng/mL	100
I	10 µL of 2,500 ng/mL	50
J	10 µL of 1,000 ng/mL	20
K	10 µL of 500 ng/mL	10
L	10 µL of 250 ng/mL	5

ANALYTICAL METHOD

Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

5.0 PREPARATION OF SPIKED STANDARDS AND BLANKS

Appropriate changes in the concentrations of the solutions may be made at the discretion of the analyst.

5.1 Multiple (e.g., about three) sets of matrix standards and a matrix blank (blank + IS) are analyzed with each set of unknown samples. A matrix double blank (blank-IS) may also be analyzed if desired.

5.2 Into individual ~20-mL culture tubes, pipet blank matrix (e.g., 0.5 mL). Pipet in the appropriate volume as described in the table above for each standard. For the blanks, pipet 10 µL of organic-free water instead of the working stock solution. Add the 10 µL of internal standard stock (~50 µg/mL) to each tube except the blank-IS (pipet 10 µL of organic-free water instead) and vortex for ~5 seconds.

5.3 For serum samples add the following to each tube: 500 µL of the TBA ion-pairing solution, 1 mL of 0.25M/0.25M carbonate/bicarbonate buffer, and 1 mL of deionized organic free water. Vortex each tube for about 5 seconds. For urine samples add the following to each tube 1 mL of TBA ion-pairing solution, 1 mL of 1.0M/1.0M carbonate/bicarbonate buffer and 1 mL of deionized water. Vortex each tube for about 5 seconds.

5.4 Add 2.5 mL of ethyl acetate and extract on horizontal mixer for 1 hour at a low speed setting.

5.5 Remove the tube from the shaker and place in a centrifuge (e.g., 2500 rpm) for about 5 minutes.

5.6 Remove the top ethyl acetate layer and put it into a clean tube and evaporate to dryness (e.g., ~ 50 minutes in the Turbo-Vap ®) with a gentle stream of nitrogen and moderate heat (e.g., 50 °C).

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Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

5.7 Reconstitute the residue in 500 µL of 5% 5 mM ammonium acetate: 95% methanol containing 1.5% formic acid and vortex briefly to mix. Filter the samples through 0.2 µm PVDF or Nylon syringe filters into autosampler vials.

6.0 PREPARATION OF SAMPLES

6.1 Allow each serum sample to thaw to room temperature. Vortex each sample briefly, but vigorously. Pipet an aliquot of each sample (e.g., 0.5 mL) into individual ~20-mL culture tubes. If necessary, dilute an aliquot of any sample with blank matrix so that the expected concentration of the test article being analyzed will fall within the concentration range of the standard curve. Add 10 µL of internal standard stock (~ 50 µg/mL) to each tube and vortex for a couple of seconds.

6.2 For serum samples add the following to each tube: 500 µL of the TBA ion-pairing solution, 1 mL of 0.25M/0.25M carbonate/bicarbonate buffer, and 1 mL of deionized organic free water. Vortex each tube for about 5 seconds. For urine samples add the following to each tube 1 mL of TBA ion-pairing solution, 1 mL of 1.0M/1.0M carbonate/bicarbonate buffer and 1 mL of deionized water. Vortex each tube for about 5 seconds.

6.3 Add 2.5 mL of ethyl acetate and extract on horizontal mixer for 1 hour at a low speed setting.

6.4 Remove the tube from the shaker and place in a centrifuge (e.g., 2500 rpm) for about 5 minutes.

6.5 Remove the top ethyl acetate layer and put it into a clean tube and evaporate to dryness (e.g., ~ 50 minutes in the Turbo-Vap ®) with a gentle stream of nitrogen and moderate heat (e.g., 50 °C).

6.6 Reconstitute the residue in 500 µL of 5% 5 mM ammonium acetate: 95% methanol containing 1.5% formic acid and vortex briefly to mix. Filter the samples through 0.2 µm PVDF or Nylon syringe filters into autosampler vials. Cap vials for analysis.

ANALYTICAL METHOD

Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

7.0 ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY/MASS SPECTROMETRY (HPLC/MS/MS)

7.1 Conditions are to be optimized if necessary.

7.1.1 HPLC Conditions

Analytical Column: Keystone Scientific Aquasil C18, 150 mm x 2 mm ID, or equivalent

Guard Column:	Keystone Aquasil C18 10 mm x 2 mm	
Elution Flow rate:	600 µL/min.	
Injection volume:	5 µL	
Mobile phase:	A: 5mM ammonium acetate buffer	
	B: 1.5% formic acid in methanol	
Gradient Profile:	0 - 3 min.	50%A : 50%B
	3 - 5min.	20% A : 80% B linear gradient
	5 - 8 min.	10%A : 90% B step gradient
	8-10 min.	50%A : 50%B step gradient
Temperature:	Ambient	

7.1.2 PE Sciex API 3000 Triple Quadrupole Mass Spectrometer Conditions
Software: PE Sciex TurboQuan

Turboion Spray Source
Note: Values listed under "MS/MS Acquisition Conditions" override parameters in this table.

Auxiliary Gas: Air (e.g., Grade 0.1) at 85 pounds per square inch

Parameter	Value
IS	-2000

ANALYTICAL METHOD

Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

Parameter	Value
NC	0
TEM	450
OR	-20
RNG	-120
Q0	10
IQ1	11
ST	15
RO1	11
IQ2	20
RO2	50
ST3	60
RO3	52
DF	250
CEM	1800
NEB	15
CUR	6
CAD	5
QPE	0
POL	1
VCM	0

ANALYTICAL METHOD

Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

Parameter	Value
IPE	0

MS/MS Acquisition Conditions
Scan type: MRM
Polarity: Negative
Acquisition mode: Profile
Pause time: 5 milliseconds

Masses requested:

PFHS:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (ms)
398.9	398.9	200

PFOC (IS)

Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (ms)
412.9	368.9	200

Parameter	Start	Stop
RO2	35	35
ST3	45	45
RO3	37	37
QO	18	18
IQ1	19	19
ST	23	23
RO1	19	19
IQ2	20	20

ANALYTICAL METHOD

Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

8.0 CALCULATIONS

8.1 At the end of the analytical run, review each chromatogram to ensure the retention time, peak shape, and peak height and peak area determination of the test article and the IS are acceptable. The data may be smoothed as appropriate. For quantitation, use the ion profiles at the following mass-to-charge ratios:

Analyte	Ion Profile
PFHS	398.9 to 398.9
PFOC	412.9 to 368.9

8.2 Plot the peak area response of PFHS divided by the peak area response of the IS (PFOC) from all standards versus the concentration of the test article in the standards. Alternatively, the peak heights may be used instead of peak areas. Obtain the best curve fit of the data (e.g., quadratic fit weighted with 1/concentration of the test article or a quadratic fit). Note: The best curve fit may be dependent on the range of the standard curve and it may be necessary to have more than one standard curve for various concentration ranges using the following:

$$y = ax^2 + bx + c$$

where
- y = Peak height response of PFHS divided by peak height response of the IS (PFOC) in standards.
- x = Concentration of the PFHS in standards.
- a, b, c = Constants derived from the regression analysis.

8.3 Using the standard curve, calculate the level of PFHS in each unknown sample. Correct the results of samples for any dilutions.

NOTE: Due to unresolvable interferences with the test article and/or internal standard from the matrix, external standard quantitation may be used at the discretion of the supervising mass spectrometrist.

ANALYTICAL METHOD

Method No.: BACG-3606

Title: Determination of Perfluorohexanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

9.0 ACCEPTANCE AND REJECTION CRITERIA

9.1 Refer to SOP SRI 91-3 for acceptance/rejection criteria except acceptable accuracy for standards is 80-120% of theoretical.

10.0 REPORTING

10.1 Results of all analyses are tabulated, and the raw data, original chromatograms, and reports are to be filed in the appropriate study file.

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4/7/03
Date

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Greg Gorman, Ph.D.
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Date

ANALYTICAL METHOD

Method No.: BACG-3607

Title: Determination of Perfluorooctanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

1.0 PRINCIPLE

Serum or urine samples are obtained from cynomolgus monkeys treated with Perfluorooctanesulfonate (PFOS). The serum or urine (e.g., 0.5 mL) containing (PFOS) is fortified with an internal standard (IS), Perfluorooctanecarboxalate (PFOC). The samples are then mixed with an ion-pairing reagent, buffer and water, followed by extraction with ethyl acetate. The ethyl acetate layer is removed, evaporated to dryness, reconstituted in 95% methanol containing 1.5 % formic acid, 5 % 5mM ammonium acetate, filtered, and transferred to autosampler vials, and analyzed by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS). The range of reliable results extends from about 20 to 10,000 ng/mL of PFOS in serum and from 10 to 500 ng/mL in urine. Samples containing PFOS at concentrations greater than 10,000 ng/mL may be diluted with control blank matrix so that the concentration of PFOS will be within the range of reliable results prior to analysis.

The mass spectrometry of PFOS and PFOC is accomplished in the negative ion mode. The ion spray source voltage is set at - 2000 volts which is low enough to greatly reduce the formation of other potentially interfering ions extracted from the matrix.

CAUTION: Since primates may carry a number of zoonoses, all unpreserved tissues, including blood, plasma and serum, are to be considered as biohazards and handled with **universal precautions.** Refer to SOP number SRI 2-5-5 for a description of safety procedures to be used when handling unpreserved primate tissue.

2.0 REAGENTS AND SOLUTIONS

The listed reagents or their equivalents may be used.

2.1 Neat Reagents

2.1.1 Water, deionized and organic free (from in-house purification system; e.g., Ingalls 210N)

2.1.2 Methanol, HPLC grade

ANALYTICAL METHOD

Method No.: BACG-3607

Title: Determination of Perfluorooctanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

2.1.3 Perfluorooctanesulfonate (analyte), as provided by the client

2.1.4 Perfluorooctanecarboxalate (internal standard), 97%

2.1.5 Ammonium acetate, HPLC grade

2.1.6 Blank control monkey serum

2.1.7 Sodium Carbonate, Certified ACS Grade or equivalent

2.1.8 Sodium Bicarbonate, Certified ACS Grade or equivalent

2.1.9 Ethyl Acetate, HPLC grade

2.1.10 Tetrabutylammonium Hydrogen Sulfate, 97%

2.1.11 Sodium Hydroxide 50% solution, Certified grade or equivalent

2.1.12 Blank control monkey urine

2.1.13 Formic Acid, 88%

2.2 **Prepared Solutions**

Appropriate changes in the solutions may be made at the discretion of the analyst

2.2.1 5 mM Ammonium acetate in organic free water

2.2.1.1 For example, to prepare 4 liters, measure out ammonium acetate (e.g., 1.542 g) and add in organic-free water (e.g., 4 L). Mix well and filter through HPLC mobile phase filtration apparatus.

2.2.2 TBA Ion-Pairing Solution (0.5 M tetrabutylammonium hydroxide)

ANALYTICAL METHOD

Method No.: BACG-3607

Title: Determination of Perfluorooctanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

2.2.2.1 For example, to prepare 25 mL, dissolve 4.24 g of tetrabutylammonium hydrogen sulfate in deionized water and adjust the pH to 10 with 50% NaOH solution.
Note: a more dilute solution of NaOH in water may be used to effect smaller pH adjustments.

2.2.3 Carbonate/Bicarbonate Buffer Solution for Serum (0.25M/0.25M)

2.2.3.1 For example, to prepare 100 mL, dissolve approximately 2.65 g of sodium carbonate and approximately 2.10 g of sodium bicarbonate in 100 mL of deionized water. Mix well to ensure complete dissolution.

2.2.4 Carbonate/Bicarbonate Buffer Solution for Urine (1.0M/1.0M)

2.2.4.1 For example, to prepare 100 mL, dissolve approximately 10.6 g of sodium carbonate and approximately 8.4 g of sodium bicarbonate in 100 mL of deionized water. Mix well to ensure complete dissolution.

3.0 INSTRUMENTS, MATERIALS, AND APPARATUS

The following or their equivalents may be used.

3.1 HPLC pump(s), autosampler, and triple quadrupole mass spectrometer

3.2 Autosampler vials with inserts

3.3 Vortex mixers (e.g., touch mixer and IKA-Vibrax ® platform mixer)

3.4 Solvent-concentration apparatus (e.g., Zymark Turbo-Vap ® with source of nitrogen)

3.5 HPLC mobile phase filtration apparatus

3.6 Filters for HPLC mobile phase filtration apparatus (e.g., Nylon-66, 0.20 µm)

ANALYTICAL METHOD

Method No.: BACG-3607

Title: Determination of Perfluorooctanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

3.7 Analytical balance

3.8 Volumetric flasks (e.g., 10 and 25 mL)

3.9 Disposable Pasteur pipettes

3.10 Micropipettor(s) with tips

3.11 Culture tubes with teflon-lined caps

3.12 Centrifuge

3.13 Assorted glassware and syringes

3.14 Culture tubes (vials) for use with solvent-concentration apparatus

3.15 1 mL Plastic syringes with 0.2 µm PVDF syringe filters

3.16 Variable speed horizontal platform shaker

3.17 pH meter

4.0 PREPARATION OF STOCKS AND WORKING STOCKS

Appropriate changes in the concentrations of the solutions may be made at the discretion of the analyst. Actual dilutions will be documented on the preparation sheets.

4.1 Main Stock Solution of PFOS ~1000 µg/mL

4.1.1 Prepare an ~1000 µg/mL solution of PFOS in deionized organic-free water (e.g., accurately weigh about 10 mg PFOS into a 10-mL volumetric flask). Add deionized organic-free water to dissolve. Dilute to the mark. Alternatively, weigh the compound

into an appropriate vessel (e.g., culture tube) and add 10 mL of deionized organic-free water. Mix well. Transfer the solution to a clean vessel if desired.

4.2 **Stock Solution of Internal Standard (PFOC), ~200 µg/mL**

4.2.1 Prepare an ~200µg/mL solution of PFOC in deionized organic-free water (e.g., accurately weigh about 10 mg into a 50-mL volumetric flask). Add deionized organic-free water to dissolve and dilute to the mark with deionized organic-free water. Alternatively, weigh the compound into a an appropriate vessel (e.g., culture tube) and add 50 mL of deionized organic-free water. Mix well. Transfer the solution to a clean vessel if desired.

4.3 **Spiking solution of Internal Standard (PFOC), ~ 50µg/mL**

4.3.1 Prepare an ~ 50 µg/mL solution of PFOC in deionized organic- free water by pipetting 2 mL of the 200 µg/mL solution into a culture tube and add 6 mL of deionized water. Mix well.

4.4 **Working Stock Solutions of PFOS**

4.4.1 To prepare working stock solutions, make the proper dilutions as shown in the following table. Prepare in 10-mL volumetric flasks or other appropriate glassware. If desired a modified dilution scheme can be used and documented in the study records.

Working Stock Level (WSL) Approximate Concentration (ng/mL)	Volume of PFOS solution	Final Volume in deionized organic free water (mL)
500,000	5 mL of 1000 µg/mL	10
250,000	5 mL of 500,000 ng/mL	10

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Title: Determination of Perfluorooctanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

62,500	2.5 mL of 250,000 ng/mL	10
31,250	5 mL of 62,500 ng/mL	10
15,625	5 mL of 31,250 ng/ mL	10
5,000	50 µL of 1000 µg/mL stock	10
2,500	5 mL of 5000 ng/mL stock	10
1,000	4 mL of 2500 ng/mL stock	10
500	5 mL of 1000 ng/mL stock	10
250	5 mL of 500 ng/mL stock	10

4.4.2 Summary of concentrations of serum standards:

Standard Level	Volume and Spike Conc.	Approximate Concentration of PFOS in serum (ng/mL)
A	10 µL of 500,000 ng/mL	10,000
B	10 µL of 250,000 ng/mL	5,000
C	16 µL of 62,500 ng/mL	2,000
D	16 µL of 31,250 ng/mL	1,000

ANALYTICAL METHOD

Method No.: BACG-3607

Title: Determination of Perfluorooctanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

E	16 µL of 15,625 ng/mL	500
F	20 µL of 5,000 ng/mL	200
G	10 µL of 5,000 ng/mL	100
H	10 µL of 2,500 ng/mL	50
I	10 µL of 1,000 ng/mL	20
J	10 µL of 500 ng/mL	10
K	10 µL of 250 ng/mL	5

5.0 PREPARATION OF SPIKED STANDARDS AND BLANKS

Appropriate changes in the concentrations of the solutions may be made at the discretion of the analyst.

5.1 Multiple (e.g., about three) sets of matrix standards and a matrix blank (blank + IS) are analyzed with each set of unknown samples. A matrix double blank (blank-IS) may also be analyzed if desired.

5.2 Into individual ~20-mL culture tubes, pipet blank matrix (e.g., 0.5 mL). Pipet in the appropriate volume as described in the table above for each standard. For the blanks, pipet 10 µL of organic-free water instead of the working stock solution. Add the 10 µL of internal standard stock (~50 µg/mL) to each tube except the blank-IS (pipet 10 µL of organic-free water instead) and vortex for ~5 seconds.

5.3 For serum samples add the following to each tube: 500 µL of the TBA ion-pairing solution, 1 mL of 0.25M/0.25M carbonate/bicarbonate buffer, and 1 mL of deionized organic free water. Vortex each tube for about 5 seconds. For urine samples add the following to each tube 1 mL of TBA ion-pairing solution, 1 mL of 1.0M/1.0M carbonate/bicarbonate buffer and 1 mL of deionized water. Vortex each tube for about 5 seconds.

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Method No.: BACG-3607

Title: Determination of Perfluorooctanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

5.4 Add 2.5 mL of ethyl acetate and extract on horizontal mixer for 1 hour at a low speed setting.

5.5 Remove the tube from the shaker and place in a centrifuge (e.g., 2500 rpm) for about 5 minutes.

5.6 Take off the top ethyl acetate layer and put it into a clean tube and evaporate to dryness (e.g., ~ 50 minutes in the Turbo-Vap ®) with a gentle stream of nitrogen and moderate heat (e.g., 50 °C).

5.7 Reconstitute the residue in 500 µL of 5% 5 mM ammonium acetate: 95% methanol containing 1.5% formic acid and vortex briefly to mix. Filter the samples through 0.2 µm PVDF or Nylon syringe filters into autosampler vials.

6.0 PREPARATION OF SAMPLES

6.1 Allow each serum sample to thaw to room temperature. Vortex each sample briefly, but vigorously. Pipet an aliquot of each sample (e.g., 0.5 mL) into individual ~20-mL culture tubes. If necessary, dilute an aliquot of any sample with blank matrix so that the expected concentration of the test article being analyzed will fall within the concentration range of the standard curve. Add 10 µL of internal standard stock (~ 50 µg/mL) to each tube and vortex for a couple of seconds.

6.2 For serum samples add the following to each tube: 500 µL of the TBA ion-pairing solution, 1 mL of 0.25M/0.25M carbonate/bicarbonate buffer, and 1 mL of deionized organic free water. Vortex each tube for about 5 seconds. For urine samples add the following to each tube 1 mL of TBA ion-pairing solution, 1 mL of 1.0M/1.0M carbonate/bicarbonate buffer and 1 mL of deionized water. Vortex each tube for about 5 seconds.

6.3 Add 2.5 mL of ethyl acetate and extract on horizontal mixer for 1 hour at a low speed setting.

ANALYTICAL METHOD

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Title: Determination of Perfluorooctanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

6.4 Remove the tube from the shaker and place in a centrifuge (e.g., 2500 rpm) for about 5 minutes.

6.5 Take off the top ethyl acetate layer and put it into a clean tube and evaporate to dryness (e.g., ~ 50 minutes in the Turbo-Vap ®) with a gentle stream of nitrogen and moderate heat (e.g., 50 °C).

6.6 Reconstitute the residue in 500 µL of 5% 5 mM ammonium acetate: 95% methanol containing 1.5% formic acid and vortex briefly to mix. Filter the samples through 0.2 µm PVDF or Nylon syringe filters into autosampler vials. Cap vials for analysis.

7.0 ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY/MASS SPECTROMETRY (HPLC/MS/MS)

7.1 Conditions are to be optimized if necessary.

7.1.1 <u>HPLC Conditions</u>

Analytical Column: Keystone Scientific Aquasil C18, 150 mm x 2 mm ID, or equivalent

Guard Column:	Keystone Aquasil C18 10 mm x 2 mm	
Elution Flow rate:	400 µL/min.	
Injection volume:	5 µL	
Mobile phase:	A: 5mM ammonium acetate buffer	
	B: 1.5% formic acid in methanol	
Gradient Profile:	0 - 3 min.	50%A : 50%B
	3 - 5min.	20% A : 80% B linear gradient
	5 - 8 min.	10%A : 90% B step gradient
	8-10 min.	50%A : 50%B step gradient
Temperature:	Ambient	

7.1.2 PE Sciex API 3000 Triple Quadrupole Mass Spectrometer Conditions
Software: PE Sciex TurboQuan

ANALYTICAL METHOD

Method No.: BACG-3607

Title: Determination of Perfluorooctanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

Turboion Spray Source

Note: Values listed under "MS/MS Acquisition Conditions" override parameters in this table.

Auxiliary Gas: Air (e.g., Grade 0.1) at 85 pounds per square inch

Parameter	Value
IS	-2000
NC	0
TEM	450
OR	-20
RNG	-120
Q0	10
IQ1	11
ST	15
RO1	11
IQ2	20
RO2	50
ST3	60
RO3	52
DF	250
CEM	1800

ANALYTICAL METHOD

Method No.: BACG-3607

Title: Determination of Perfluorooctanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

Parameter	Value
NEB	15
CUR	6
CAD	5
QPE	0
POL	1
VCM	0
IPE	0

MS/MS Acquisition Conditions
Scan type: MRM
Polarity: Negative
Acquisition mode: Profile
Pause time: 5 milliseconds

Masses requested:

PFOS:

Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (ms)
498.9	498.9	200

PFOC (IS)

Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (ms)
412.9	368.9	200

Parameter	Start	Stop
RO2	35	35
ST3	45	45
RO3	37	37
QO	18	18
IQ1	19	19
ST	23	23

ANALYTICAL METHOD

Method No.: BACG-3607

Title: Determination of Perfluorooctanesulfonate in Monkey Serum and Urine: Sample Preparation and Analysis by HPLC Mass Spectrometry/Mass Spectrometry (HPLC/MS/MS)

RO1	19	19
IQ2	20	20

8.0 CALCULATIONS

8.1 At the end of the analytical run, review each chromatogram to ensure the retention time, peak shape, and peak height and peak area determination of the test article and the IS are acceptable. The data may be smoothed as appropriate. For quantitation, use the ion profiles at the following mass-to-charge ratios:

Analyte	Ion Profile
PFOS	498.9 to 498.9
PFOC	412.9 to 368.9

8.2 Plot the peak area response of PFOS divided by the peak area response of the IS (PFOC) from all standards versus the concentration of the test article in the standards. Alternatively, the peak heights may be used instead of peak areas. Obtain the best curve fit of the data (e.g., quadratic fit weighted with 1/concentration of the test article or a quadratic fit). Note: The best curve fit may be dependent on the range of the standard curve and it may be necessary to have more than one standard curve for various concentration ranges using the following:

$$y = ax^2 + bx + c$$

where y = Peak height response of PFOS divided by peak height response of the IS (PFOC) in standards.
x = Concentration of the PFOS in standards.
a, b, c = Constants derived from the regression analysis.

ANALYTICAL METHOD

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8.3 Using the standard curve, calculate the level of PFHC in each unknown sample. Correct the results of samples for any dilutions.

NOTE: Due to unresolvable interferences with the test article and/or internal standard from the matrix, external standard quantitation may be used at the discretion of the supervising mass spectrometrist.

9.0 ACCEPTANCE AND REJECTION CRITERIA

9.1 Refer to SOP SRI 91-3 for acceptance/rejection criteria except acceptable accuracy for standards is 80-120% of theoretical.

10.0 REPORTING

10.1 Results of all analyses are tabulated, and the raw data, original chromatograms, and reports are to be filed in the appropriate study file.

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