AR 226_3313

TRADE SECRET

Study Title

H-25509: Repeated-Dose Dermal Toxicity
28-Day Study in Male Rats
Supplement Number 1

TEST GUIDELINES: U.S. EPA Health Effects Test Guidelines
OPPTS 870.3200 (1998)

OECD Guidelines for Testing of Chemicals
Section 4: Health Effects, Number 410 (1981)

AUTHOR: Carol Finlay, B.A.

STUDY COMPLETED ON: August 4, 2003

SUPPLEMENT NUMBER 1 COMPLETED ON: January 14, 2004

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
Haskell Laboratory for Health and Environmental Sciences
Elkton Road, P.O. Box 50
Newark, Delaware 19714-0050

LABORATORY PROJECT ID: DuPont-11763

[redacted]

Company Sanitized. Does not contain TSCA CBI

QUALITY ASSURANCE STATEMENT

Haskell Sample Number(s):

25509

Dates of Inspections:

Supplement Number 1: December 12,15, 2003

Dates Findings Reported to:

Study Director: December 15,17, 2003

Management: December 15,17, 2003

Reported by: Wonda K. Kelly 14-Jan-2004

Wonda K. Kelly
Quality Assurance Auditor

Date

CERTIFICATION

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

Approved by: _____ 13 JAN 2004
Scott E. Loveless, Ph.D. — Date
Management

Issued by Study Director: _____ 14 Jan 2004
Carol Finlay, B.A. — Date
Staff Toxicologist

Company Sanitized. Does not contain TSCA CBI

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STUDY INFORMATION

Substance Tested: [redacted]

Synonyms/Codes: [redacted]

Submitter's Notebook Number(s): [redacted]

Haskell Number: 25509

[redacted]

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

Study Initiated/Completed: 22-Nov-2002 / (see report cover page)

REASON FOR SUPPLEMENT NUMBER 1

This supplement contains the results of the analyses of plasma samples collected at terminal sacrifice and analyzed for the presence of [redacted]

SUMMARY

A 28-day dermal toxicology study was conducted with male Crl:CD®(SD)IGS BR rats. Ten rats per group were exposed dermally for 6 hours/day for a total of 28 consecutive days to dosages of 0, 5, 50, or 500 mg/kg/day H-25509. The ground test material was mixed with mineral oil, applied to the shaved, intact skin of the rats, and covered with occlusive dressings for the duration of the daily exposure. All rats were sacrificed at the end of the exposure period. Under the conditions of the study, the NOEL for toxicity was 50 mg/kg/day, based on decreased cholesterol at 500 mg/kg/day.

In addition to assessments for toxicology endpoints, blood was collected for analyses at the end of the exposure period. Whole blood was collected on study day 27 and retained for analysis of total fluorine. The whole blood was analyzed by use of a Wickbold torch combustion method and an ion selective electrode. The limit of detection (LOD) for the method is 0.5 ppm (500 ppb).

At the 28-day sacrifice, blood was collected, centrifuged, and plasma retained for [redacted] analysis [redacted] was extracted from plasma by protein precipitation in acetonitrile. The compound was quantified by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis using selected reaction monitoring (SRM). Quantification was performed using extracted calibration standards containing an internal standard. The lower limit of quantitation (LLOQ) for this method is 10 ppb. Results of these analyses are presented in this supplemental report.

One sample each of the stock mineral oil and stock dressing material used to occlude the test sample application was also analyzed for the presence of total fluorine. The Wickbold torch combustion method was used for this analysis.

The [redacted] levels in plasma were 61.4 ppb, 155.5 ppb, 1315.9 ppb, and 14176 ppb for dosages of 0, 5, 50, and 500 mg/kg/day, respectively. The total fluorine levels in blood were 1075 ppb, 1470 ppb, and 9316 ppb for dosages of 0, 50, 500 mg/kg/day, respectively. All total fluorine levels at 5 mg/kg/day were below the limit of detection. There was 500 ppb fluorine in the stock mineral oil and 1760 ppb fluorine in the stock bandage material.

The results of the analysis o[redacted] are summarized in Table 1 (based on Appendix A, Table III of Exygen report). The results of the analysis of total fluorine in the mineral oil dressing material are in Table 1. The analyses of total fluorine were in the original report and have been included in Table 1 for comparative purposes. The details of the analyses o[redacted], which were conducted by Exygen Research, are reported in Appendix A.

TABLE 1

MEASURED CONCENTRATION (ppb)
Blood Analyses

Dosage (mg/kg/day)	WHOLE BLOOD FLUORINE[a] MEAN	S.D.	n	PLASMA [redacted] MEAN	S.D.	n
0	1075	700.0	5	61.4	27.9	10
5	<500*	0.0	5	155.5	25.5	10
50	1470	308.6	5	1315.9	482.3	10
500	9316	5742.4	5	14176	4534.0	10

N=number of samples
S.D. = Standard deviation
* All values below limit of detection.
a LOD = 500 ppb
b LLOQ = 10 ppb

SAMPLE ANALYSIS

SAMPLE	TOTAL FLUORINE (ppb)
Mineral Oil	500
Bandage Material	1760

APPENDIX A

Analytical Report

STUDY TITLE

H-25509: Repeated-Dose Dermal Toxicity 28-Day Study in Male Rats

DATA REQUIREMENTS

Analytical Method Requirements

STUDY DIRECTOR

Carol Finlay

ANALYTICAL PHASE COMPLETED ON

September 23, 2003

PERFORMING LABORATORY / TESTING FACILITY

Exygen Research
3058 Research Drive
State College, PA 16801
Phone: 814-272-1039

STUDY SPONSOR

E. I. du Pont de Nemours and Company (DuPont)
P.O. Box 50
Newark, DE 19714-0050 USA

PROJECT

[redacted]

Sponsor Study No.: DuPont-11763

Total Pages: 72

[redacted]

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

[redacted] entitled "H-25509: Repeated-Dose Dermal Toxicity 28-Day Study in Male Rats," conducted for DuPont, was performed in compliance with US EPA TSCA (40 CFR part 792) by Exygen Research.

Emily R Decker
Emily R. Decker
Principal Investigator
Exygen Research

9/23/03
Date

Carol Finlay
Carol Finlay
Study Director
DuPont

14-Oct-2003
Date

S. Mark
S. Mark Kennedy
Sponsor Representative
DuPont

9-Oct-2003
Date

[redacted]

QUALITY ASSURANCE STATEMENT

Exygen Research's Quality Assurance Unit reviewed [redacted] entitled, "H-25509: Repeated-Dose Dermal Toxicity 28-Day Study in Male Rats". All phases were reviewed for conduct according to Exygen Research's Standard Operating Procedures, the Study Protocol, and all applicable Good Laboratory Practice Standards. All findings were reported to the Study Director and to management.

Phase	Date Inspected	Date Reported to Principal Investigator	Date Reported to Exygen Management	Date Reported to Study Director and Sponsor Management
1. Fortification and Extraction	07/23/03	07/29/03	08/13/03	08/19/03
2. Raw Data & Draft Report Review	08/11,12/03	08/14/03	08/16/03	08/19/03
3. Raw Data & Final Analytical Report Review	09/22/03	09/22/03	09/23/03	09/23/03

Lydia J. Shaffer
Lydia J. Shaffer
Quality Assurance Auditor

09/23/03
Date

[redacted]

CERTIFICATION OF AUTHENTICITY

This report, for [redacted] is a true and complete representation of the raw data for the study.

Submitted by: Exygen Research
3058 Research Drive
State College, PA 16801
(814) 272-1039

Principal Investigator, Exygen:

Emily R. Decker — 9/23/03
Emily R. Decker — Date
Scientist/Principal Investigator
Exygen Research

Exygen Research Facility Management:

John M. Flaherty — 9/23/03
John M. Flaherty — Date
Vice President
Exygen Research

Study Director, DuPont:

Carol Finlay — 14. Oct. 2003
Carol Finlay — Date
DuPont

Sponsor Representative, DuPont:

S. Mark Kennedy — 9-OCT-2003
S. Mark Kennedy — Date
DuPont

[redacted]

STUDY IDENTIFICATION

H-25509: Repeated-Dose Dermal Toxicity 28-Day Study in Male Rats

SPONSOR STUDY NUMBER:	DuPont-11763
[redacted]	
TYPE OF STUDY:	Residue
SAMPLE MATRIX:	Rat Plasma
TEST SUBSTANCE:	[redacted]
SPONSOR:	E. I. du Pont de Nemours and Company P.O. Box 50 Newark, DE 19714-0050 Phone: (302) 366-5255
STUDY DIRECTOR:	Carol Finlay DuPont
TESTING FACILITY:	Exygen Research 3058 Research Drive State College, PA 16801
ANALYTICAL PHASE TIMETABLE:	Study Initiation Date: 11/22/02 Analytical Start Date: 07/22/03 Analytical Termination Date: 07/31/03 Analytical Report Completion Date: xx/xx/xx

[redacted]

PROJECT PERSONNEL

The Study Director for this project at DuPont was Carol Finlay. The following personnel from Exygen Research were associated with various phases of the study:

Name	Title
Paul Connolly	Technical Lead-LC/MS
Emily Decker	Scientist
Mark Neeley	Scientist
Mark Ammerman	Sample Custodian

[redacted]

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LIST OF TABLES

[redacted]

LIST OF FIGURES

LIST OF APPENDICES

[redacted]

1.0 SUMMARY

Exygen Research (Exygen) analyzed samples of rat plasma for residues of [redacted] in according to protocol DuPont-11763 (**Appendix A**) using the analytical method [redacted] **Appendix B**).

The limit of quantitation for this method is 10 ng/mL, which was established at Exygen in the method validation study [redacted]

Residues of [redacted] in the rat plasma samples ranged from 19.3 ng/mL to 26,000 ng/mL.

The average recovery ± standard deviation for [redacted] in fortified rat plasma samples was 101% ± 4.6%.

2.0 OBJECTIVE

The objective of this analytical phase was to analyze rat plasma samples received at Exygen using the method [redacted] entitled "Method of Analysis for the Determination of [redacted] in Serum by LC/MS/MS".

3.0 INTRODUCTION

This report details the results of the analysis of rat plasma samples for [redacted] using the analytical method [redacted]

The study DuPont-11763 was initiated on November 22, 2002, when the study director signed protocol number 1012. The analytical start date was July 22, 2003, and the analytical termination date was July 31, 2003.

4.0 TEST SYSTEM

The control rat plasma used for control samples and laboratory control fortified samples was received from DuPont. Forty rat plasma samples were received at Exygen on July 22, 2003, logged in by Exygen personnel and placed in frozen storage (≤ -20°C).

Sample login and chain of custody information can be found in the raw data package associated with this study. Storage records will be kept at Exygen Research and a true copy of the storage records will be furnished upon request.

[redacted]

5.0 REFERENCE MATERIAL

The analytical standard [redacted] was received at Exygen on October 9, 2002 from Oakwood Products, Inc. The control article (internal standard) [redacted] was received at Exygen on February 20, 2003 from DuPont.

The available information for the reference material is listed below. The analytical standard was stored dessicated in a refrigerator and the control article was stored at room temperature.

Compound	Exygen Inventory No.	Lot No.	Purity (%)	Expiration Date
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

The structure of [redacted] and the internal standard are given below.

[redacted]

[redacted]

6.0 DESCRIPTION OF ANALYTICAL METHOD

6.1 Extraction Procedure

1. Measure 0.1 mL of sample into 2 mL eppendorf centrifuge tubes (fortify with analyte as needed, close lid, and vortex ~ 10 seconds).
2. Add enough acetonitrile containing internal standard at 0.005 µg/mL (accounting for fortification volume) to make extraction volume 500 µL and vortex for ~ 10 seconds.
3. Centrifuge for ~ 10 minutes at ~ 14,000 rpm.
4. Analyze samples using electrospray LC/MS/MS.

6.2 Preparation of Standards and Fortification Solutions

The stock standard solution of [redacted] was prepared on May 21, 2003, at a concentration of approximately 100 µg/mL by dissolving approximately 10 mg of the standard (corrected for purity) in methanol. The stock standard solution of [redacted] was prepared on March 17, 2003, at a concentration of approximately 123 µg/mL by dissolving approximately 10 mg of the standard (corrected for purity) in methanol. A fortification solution of [redacted] at 10 µg/mL was prepared by taking 10 mL of the stock standard solution and bringing the volume up to 100 in acetonitrile. A 0.98 µg/mL fortification solution of [redacted] was prepared by taking 0.8 mL of the stock and bringing the volume up to 100 mL with acetonitrile.

A set of standards containing [redacted] with internal standards was prepared by dilution of the 10.0 µg/mL [redacted] solution and the 0.98 µg/mL [redacted] solution in the following manner. These solutions were used to fortify the samples and the extracted calibration standards.

Initial Conc. (µg/mL)[1]	Volume (mL)	Diluted to (mL)	Final Conc. (µg/mL)[1]
10.0	10.0	100[2]	1.0
10.0	1.0	100[2]	0.1
10.0	0.1	100[2]	0.01

[1] of [redacted] only

[2] 0.5 mL of the 0.98 µg/mL [redacted] solution was added prior to making final volume to give a concentration of 0.005 µg/mL of [redacted] in each solution.

The calibration standards are processed through the extraction procedure, identical to the samples. The extracted standards are assigned a two-week expiration date from the date of extraction based on refrigerator stability data obtained during the method validation

[redacted]

study ([redacted] The fortification of the standards before extraction is done according to the following table:

Conc. Of [redacted] Fortification Solution (µg/mL)	Fortification Volume (µL)	Volume of Control Sample (mL)	Conc. of Extracted Calibration Standard (ppb)
0.01	100	0.1	10
0.01	200	0.1	20
0.1	50	0.1	50
0.1	100	0.1	100
0.1	200	0.1	200

The extraction solution was prepared by taking 0.5 mL of the 0.98 µg/mL [redacted] solution and bringing the volume up to 100 mL with acetonitrile. This solution was also used for the dilution solvent.

The stock standard solution and all fortification/calibration standard solutions were stored in a refrigerator (4° ± 2°C) when not in use. Documentation of standard preparation can be found in the raw data associated with this report.

6.3 Chromatography

Quantification of [redacted] was accomplished by analysis using electrospray LC-MS/MS. The retention time of [redacted] was ~ 2.0 min., with no significant interfering peaks (< 20% of the LOQ standard) in the control samples corresponding to the analyte retention times.

6.4 Instrument Sensitivity

The smallest amount of [redacted] injected during the chromatographic run was equivalent to 10 ng/mL.

6.5 Description of Instrument and Operating Conditions

A PE Sciex LC-MS/MS coupled to a Hewlett Packard HPLC system was used. Data acquisition and processing were performed using Analyst 1.2 software.
Detailed operating conditions are listed below:

Mass Spec:	PE SCIEX API 4000 Biomolecular Mass Analyzer
Interface:	SCIEX Turbo Ion Spray Liquid Introduction Interface Turbo Ion Spray temperature = 350° C with N2 at ~7 L/min
Computer:	Dell Optiplex GX110
Software:	PE Sciex Analyst: Version 1.2

[redacted]

HPLC: Hewlett Packard (HP) Series 1100
HP Quat Pump
HP Vacuum Degasser
HP Autoinjector
HP Column Compartment

HPLC Column: Genesis C_8 (Jones Chromatograhpy), 2.1 mm x 50 mm, 4µ
Column Temp.: 30° C
Mobile Phase (A): 2 mM Ammonium Acetate in water
Mobile Phase (B): Methanol

Time	% A	% B	Flow (mL/min)
0	40	60	0.3
3.0	40	60	0.3
3.5	0	100	0.3
3.7	0	100	0.5
7.0	0	100	0.5
7.5	40	60	0.5
9.0	40	60	0.5
9.5	40	60	0.3
12.0	40	60	0.3

Injection Vol.: 5 µL

Ions monitored:

Analyte	Mode	Transition Monitored
[redacted]	Negative	413 → 369
[redacted]	Negative	415 → 370

6.6 Quantitation and Example Calculation

Five microliters of sample or calibration standard were injected into the LC-MS/MS. The peak area was measured and the standard curve was generated by linear regression using 1/x weighting of the ratio analyte peak area/internal standard peak area versus the ratio of the concentration of analyte/concentration of internal standard using Analyst 1.2 (or equivalent) software system. The residue concentration fo[redacted] was determined from the following equations:

Equation 1 was used to calculate the amount of analyte found (in ppb or ng/mL, based on ratio of analyte peak area to internal standard peak area) using the standard curve (linear regression parameters) generated by the Analyst software program.

Equation 1:

$$\text{Analyte found (ppb)} = \frac{(\text{Analyte Peak area/IS peak area}) - \text{intercept})}{\text{slope}} \times \text{IS conc. (ng/mL)} \times \text{DF}$$

[redacted]

For samples fortified with known amounts of analyte prior to extraction, Equation 2 was used to calculate the percent recovery.

Equation 2:

$$\text{Recovery (\%)} = \frac{\text{Analyte found (ppb)} - \text{avg. Analyte found in control (ppb)}}{\text{Amount Analyte added (ppb)}} \times 100$$

An example of a calculation using an actual sample follows: Rat plasma sample Exygen [redacted] fortified at 10 ppb with [redacted].

Where:

analyte peak area	=	79538
IS peak area	=	172676
IS conc. (ng/mL)	=	4.167 ng/mL
intercept	=	0.0344438
slope	=	0.179697
dilution factor (DF)	=	1
ppb added (fort level)	=	10
amt found in control	=	< 10 (Not quantifiable)

From equation 1:

$$\text{Analyte found (ppb)} = \frac{[(79538/172676) - 0.0344438] \times 4.167 \times 1}{0.179697}$$

$$= 9.88 \text{ ppb}$$

From equation 2:

$$\text{\% Recovery} = \frac{(9.88 \text{ ppb} - 0)}{10 \text{ ppb}} \times 100$$

$$= 99\%$$

Note: This example calculation was done using rounded numbers, and therefore may be slightly different from the values shown in the RAW DATA.

7.0 EXPERIMENTAL DESIGN

Each analytical set was extracted according to method [redacted] and consisted of a reagent blank (except for [redacted]) one matrix control, two laboratory matrix

[redacted]

controls fortified at known concentrations, one field sample fortified at a known concentration, ten to twenty field samples, and one field sample extracted in duplicate.

8.0 RESULTS

The [redacted] found in the control rat plasma samples are listed in **Table I.**

Individual recoveries for [redacted] in the laboratory fortified rat plasma samples are detailed in **Table II.** The average percent recoveries ± standard deviation for [redacted] in the rat plasma samples was 101% ± 4.6%.

Individual results for the [redacted] in the rat plasma samples are given in **Table III.** At the request of the sponsor, ten rat plasma samples were re-extracted to confirm the residue detected. The results for the re-extracted samples are given in **Table IV.** Residues of [redacted] in the rat plasma samples ranged from 19.3 ng/mL to 26,000 ng/mL.

A typical calibration curve for [redacted] and representative chromatograms are given in **Figures 1-5.**

9.0 CIRCUMSTANCES THAT MAY HAVE AFFECTED THE QUALITY OR INTEGRITY OF THE DATA

There are no known circumstances that have affected the quality or integrity of the data.

10.0 CONCLUSIONS

The rat plasma samples were successfully analyzed according to method [redacted]

11.0 RETENTION OF DATA AND SAMPLES

When the final report is complete, all original paper data generated by Exygen Research will be shipped to the sponsor. This does not include facility-specific raw data such as instrument logs. Exact copies of all raw data, as well as a signed copy of the final analytical report and all original facility-specific raw data, will be retained in the Exygen Research's archives for the period of time specified in 40 CFR Part 792. Retained samples of reference substances are archived by the sponsor.

[redacted]

TABLES

[redacted]

Table I. Summary of [redacted] (ng/mL) in Rat Plasma Control Samples

Sponsor ID	[redacted] (ng/mL)
#17 Control 07/22/03	< 10.0
#17 Control 07/23/03	< 10.0
#18 Control 07/31/03	< 10.0

[redacted]

Table II. Summary of Recoveries of [redacted] (%) in Rat Plasma Samples

Sponsor ID	[redacted] (ng/mL)	Amount Fortified (ng/mL)	Recovery (%)
#17 (LCS A) 07/22/03	9.88	10.0	99
#17 (LCS B) 07/22/03	1050	1000	105
667165 (Matrix Spk)	1220	1000	109
#17 (LCS A) 07/23/03	10.2	10.0	102
#17 (LCS B) 07/23/03	1000	1000	100
667185 (Matrix Spk)	2970	1000	95
#18 (LCS A) 07/31/03	10.3	10	103
#18 (LCS B) 07/31/03	97.0	100	97
667166 (Matrix Spk)	184	100	96
		AVERAGE:	101
		STD DEV:	4.6
		RSD:	4.5

[redacted]

Table III. Summary of Residue Found for [redacted] (ng/mL) in Rat Plasma Samples

Sponsor ID	PFOA (ng/mL)
667165	132
667165 Dup	118
667166	79.7
667167	65.6
667168	75.3
667169	40.4
667170	37.8
667171	36.4
667172	38.5
667173	68.6
667174	46.7
667175	155
667176	165
667177	111
667178	179
667179	197
667180	161
667181	132
667182	134
667183	175
667184	146
667185	2020
667185 Dup	2050
667186	999
667187	958
667188	1220
667189	973
667190	1940
667191	1050
667192	2040
667193	965
667194	979
667195	12500
667196	16100
667197	11300
667198	9660
667199	26000
667200	13600
667201	13000
667202	13300
667203	14800
667204	11500

[redacted]

Table IV. Summary of Residue Found for [redacted] (ng/mL) in Re-Extracted Rat Plasma Samples

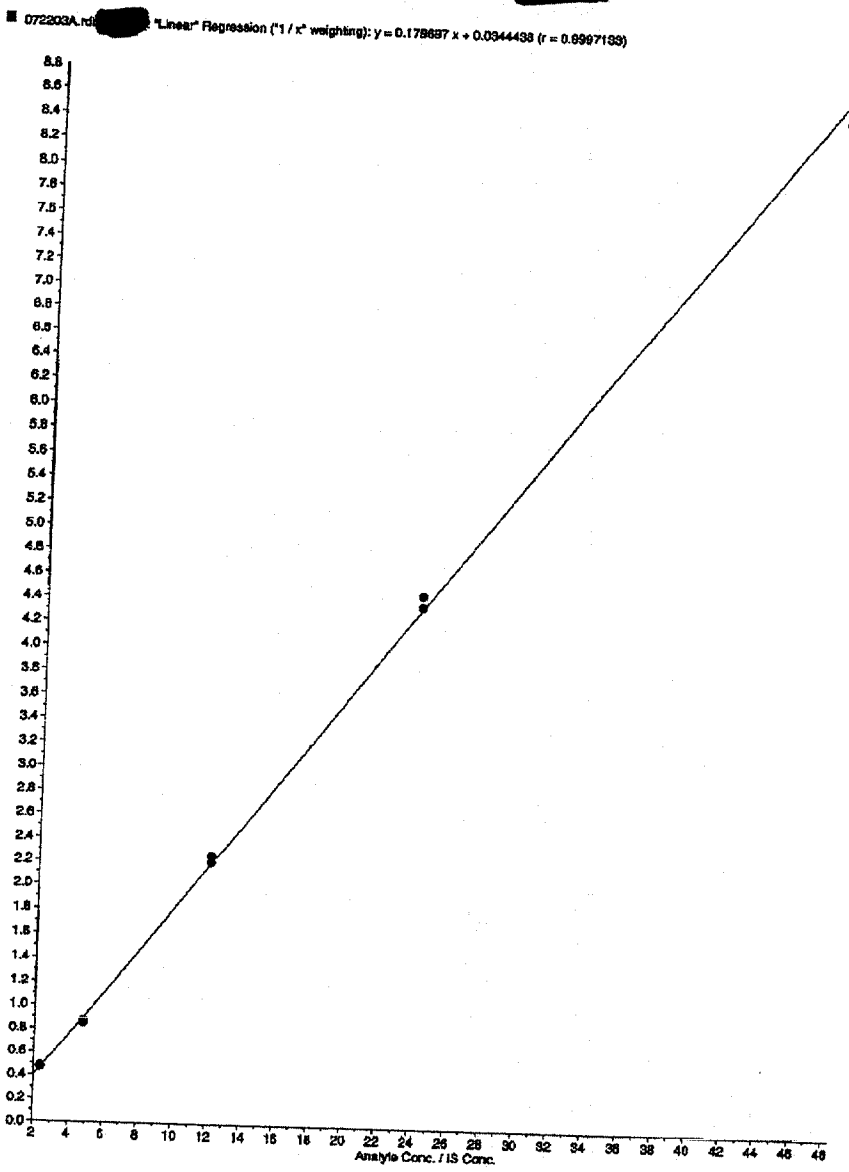
Sponsor ID	[redacted] (ng/mL)
667165	94.7
667166	88.6
667166 Dup	86.8
667167	64.9
667168	73.9
667169	40.6
667170	37.3
667171	19.3
667172	38.2
667173	64.3
667174	47.4

[redacted]

FIGURES

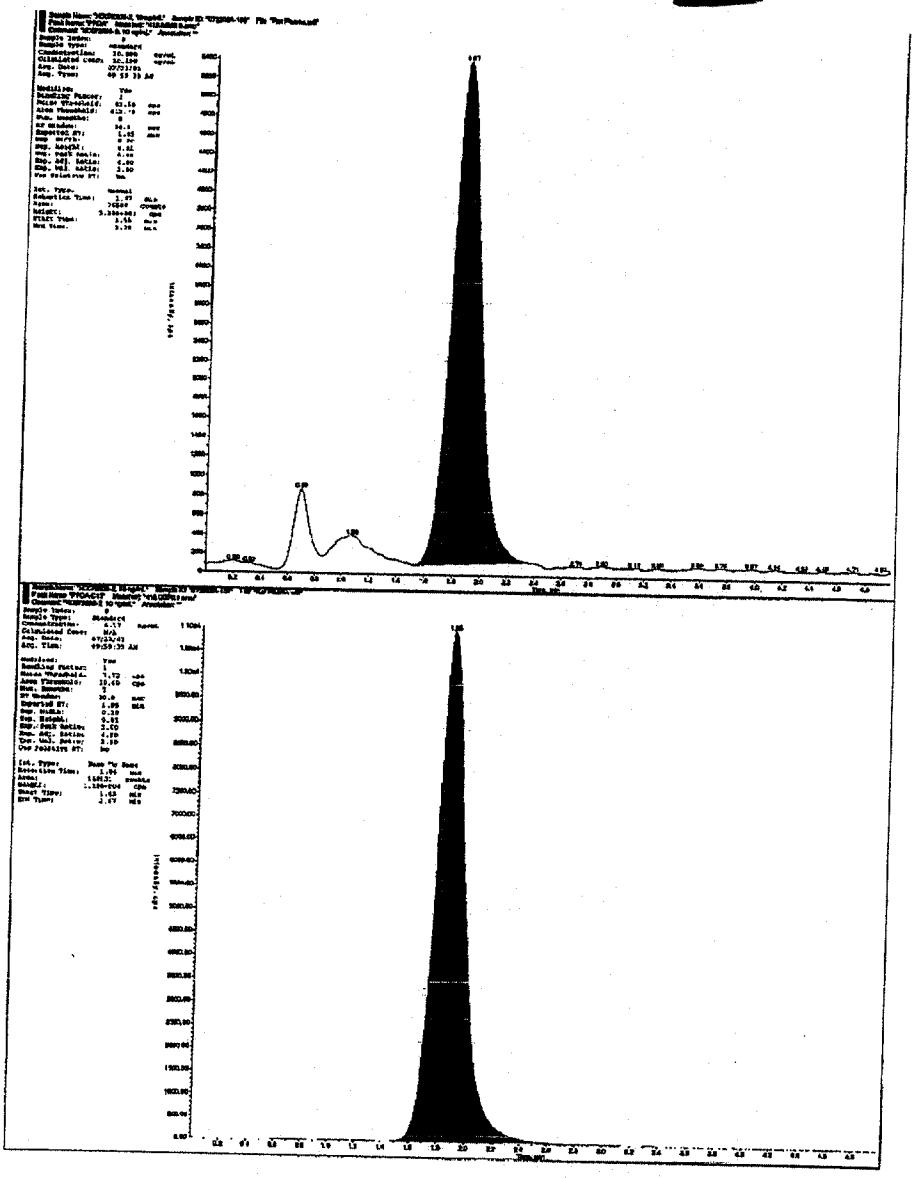
[redacted]

Figure 1. Typical Calibration Curve for [redacted]



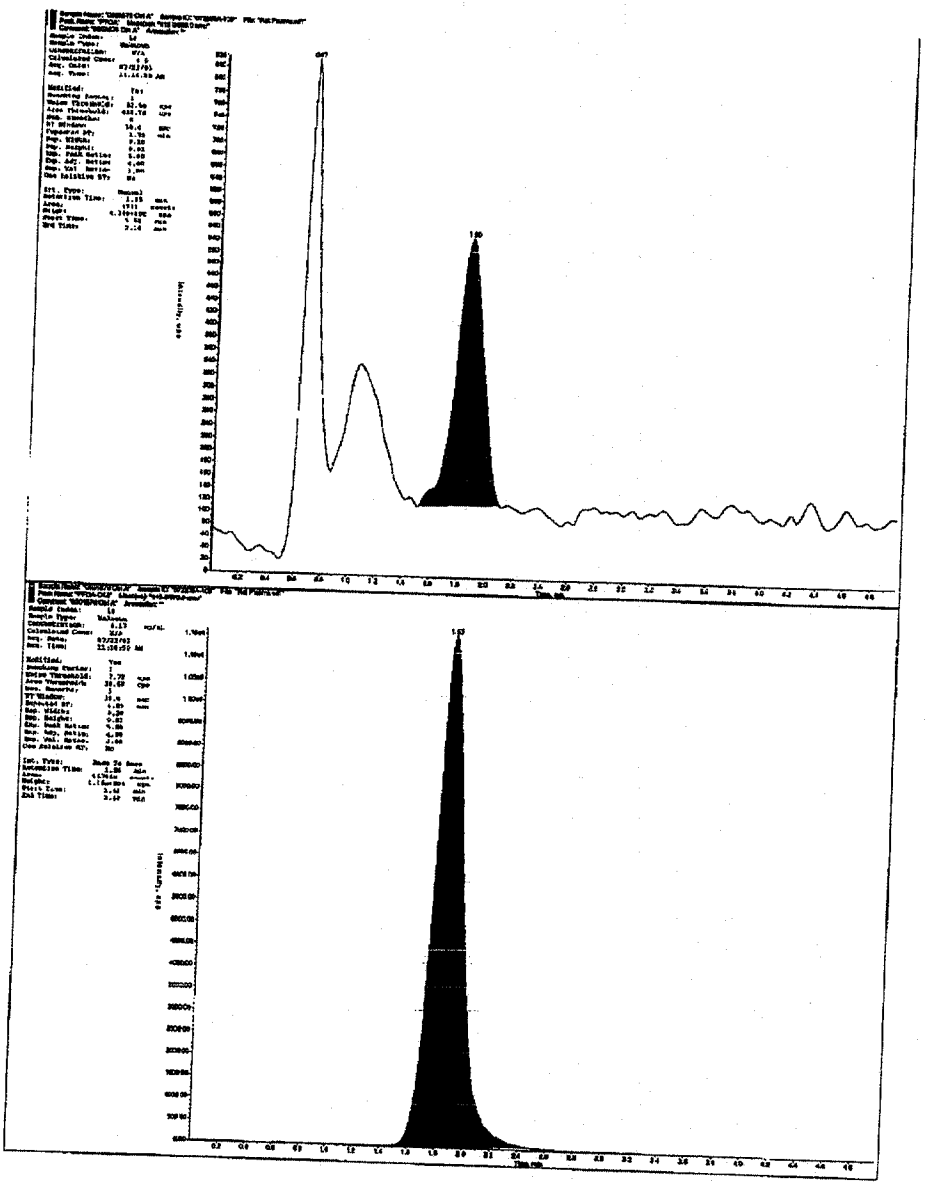
[redacted]

Figure 2. Chromatogram Representing a Calibration Standard for [redacted] at 10 ppb with ~ 5 ng/mL of ^{13}C-[redacted]



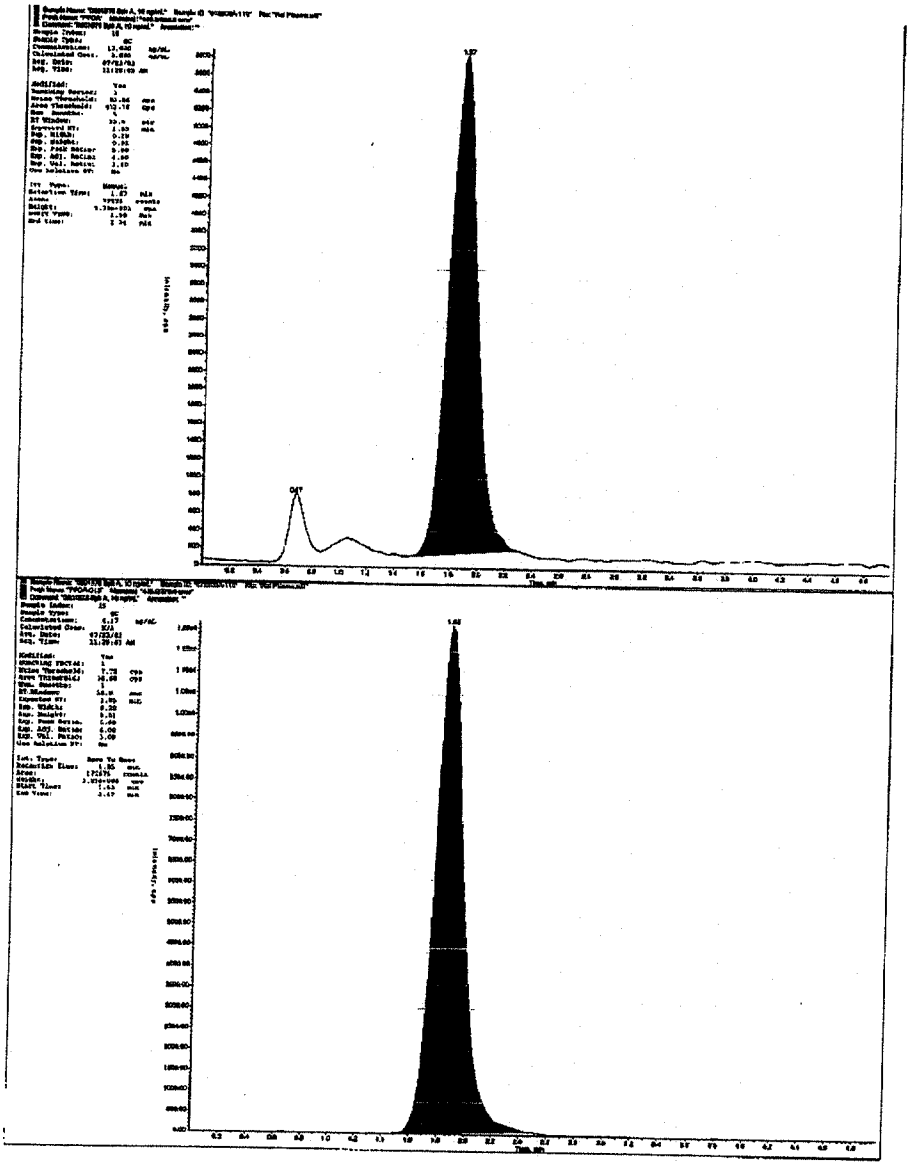
[redacted]

Figure 3. Chromatogram Representing Control Rat Plasma for [redacted] with ~ 5 ng/mL of ^{13}C-[redacted]



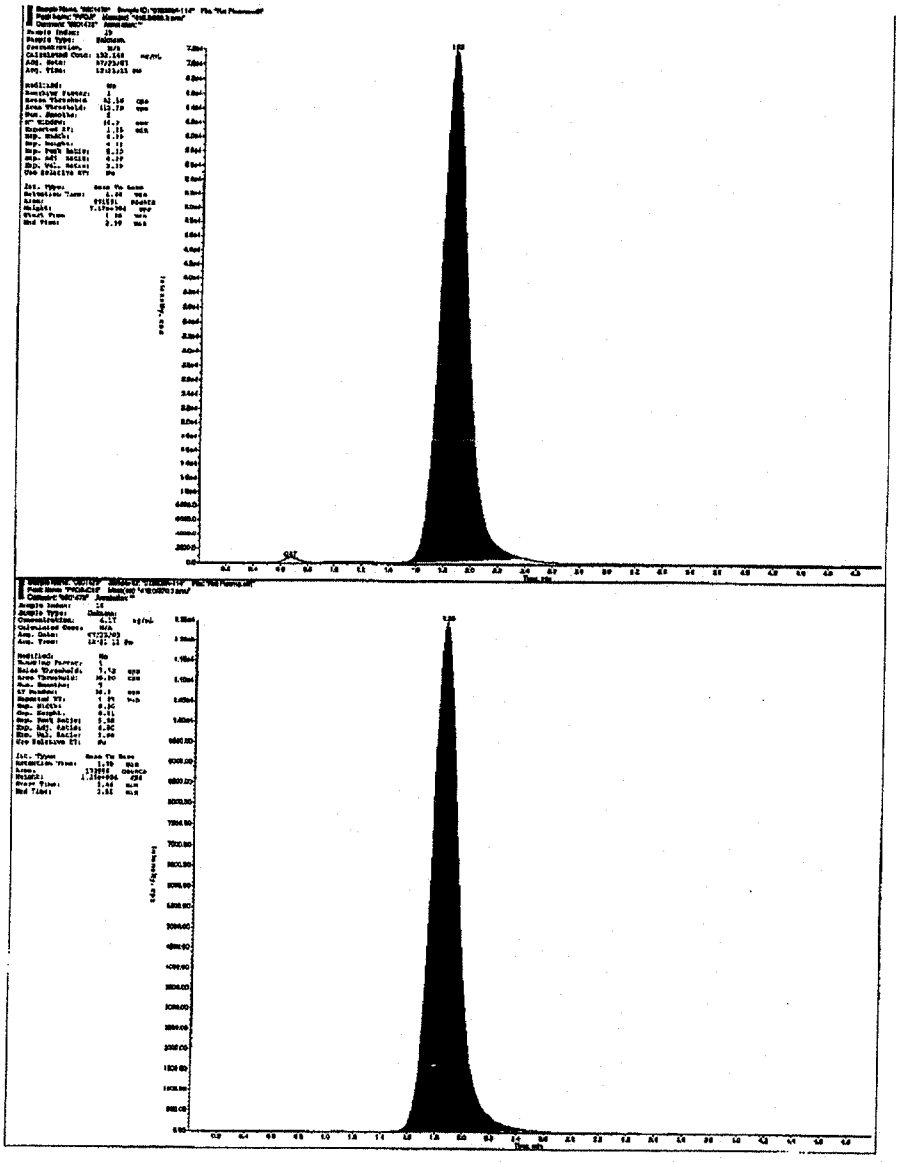
[redacted]

Figure 4. Chromatogram Representing Control Rat Plasma Fortified at 10 ppb with [redacted] and ~ 5 ng/mL of ^{13}C [redacted]



[redacted]

Figure 5. Chromatogram Representing Rat Plasma Sample for [redacted] with ~ 5 ng/mL of ^{13}C [redacted]



[redacted]

APPENDIX A

Study Protocol
DuPont-11763
[redacted]
and
Amendments and Deviation

[redacted]

JUL. 23. 2003 6:17AM HASKELL 1 / 1300 NO. 6286 P. 2/23

DuPont-11763

**H-25509: Repeated-Dose Dermal Toxicity
28-Day Study in Male Rats**

[redacted]

Protocol

Haskell Animal Welfare Committee Number: [redacted]

RECEIVED TIME JUL.23. 6:26AM PRINT TIME JUL.23. 6:40AM

[redacted]

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[redacted]

INTRODUCTION

The test substance contains an organofluoride melt additive and is used as a protectant for textiles. The dosages of the test substance were selected on the basis of a range finding study.

OBJECTIVE

The objective of this study is to evaluate the subchronic toxicity potential of H-25509 administered dermally to male rats for 28 days. The dermal route of administration was selected because it is a potential route of human exposure.

SPONSOR AND TEST FACILITY

This study is sponsored by E.I. du Pont de Nemours and Company, Wilmington, Delaware. The sponsor's approval was effective the date the sponsor authorized the work on the Work Authorization Form.

The study will be conducted at Haskell Laboratory for Health and Environmental Sciences, E.I. du Pont de Nemours and Company, Newark, Delaware.

REGULATORY COMPLIANCE

This study will be conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are consistent with the OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM(98)17.[1,2] The study design is based on U.S. Environmental Protection Agency (EPA) Office of Prevention, Pesticides, and Toxic Substances (OPPTS),[3] Organization for Economic Cooperation and Development (OECD),[4] test guidelines. Areas of noncompliance will be documented in the final report.

[redacted]

STUDY DESIGN

The study design is as follows:

Group	Number/Group	Dosage (mg/kg)
I	10	0 (Control)
III	10	5
V	10	50
VII	10	500

Study Parameters	Frequency
Body Weight	Test day 0 and at 3-4 day intervals thereafter
Food Consumption	Test day 0 and weekly thereafter
Clinical Observations	Daily
Observations for dermal effects	Daily
Mortality/Moribundity Checks	Twice daily
Blood collection for Total Fluorine Analysis	Test days 0, 7, 14, 27
Clinical Pathology	Week 4
[redacted]lood analysis	Week 4
Necropsy	Week 4

MATERIALS AND METHODS

A. Test Substance

The test substance was supplied by the sponsor and was assigned the unique Haskell Laboratory Number 25509.

B. Test Species

Male Crl:CD®(SD)IGS BR rats will be obtained from Charles River Laboratories, Inc. The location of the supplier (city/state) will be documented in the study records and final report. The Crl:CD®(SD)IGS BR rat has been selected on the bases of extensive experience with this strain at Haskell Laboratory and its suitability with respect to hardiness, longevity, sensitivity, and low incidence of spontaneous diseases.

[redacted]

C. Animal Husbandry

All rats will be housed in stainless steel, wire-mesh cages suspended above cage boards. Animal rooms will be maintained at a temperature of 22 ± 3°C and a relative humidity of 50 ± 20%. Animal rooms will be artificially illuminated (fluorescent light) on an approximate 12-hour light/dark cycle.

All rats will be provided tap water *ad libitum*. All rats will be fed PMI® Nutrition International, LLC Certified Rodent LabDiet® 5002 *ad libitum*.

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. Data are maintained separately from study records and may be included in the final report at the discretion of the study director.

D. Pretest Period

Upon arrival at Haskell Laboratory, all rats will be housed one per cage in quarantine. The rats will be:

- quarantined for a minimum of 5 days.
- identified temporarily by cage identification.
- weighed at least 3 times during quarantine.
- observed with respect to weight gain and any gross signs of disease or injury.

The rats will be released from quarantine by the laboratory animal veterinarian or designee on the bases of body weights and clinical signs.

[redacted]

Rats that are accidentally killed or removed from study during the pretest period will be discarded without necropsy. Rats that are found dead or sacrificed *in extremis* during the pretest period will be sent to Pathology and given a gross examination to check for the presence of disease. Dependent upon these findings, further diagnostic procedures may be employed at the discretion of the study director, a pathologist, or the laboratory animal veterinarian. The results will not be reported in the final report unless considered significant to the evaluation of the study.

E. Range Finding Study

A range finding study was conducted to aid in dosage selection. The test substance, moistened with mineral oil, was applied to the shaved, intact skin of groups of two male rats at a dosage of 10, 100, or 1000 mg/kg for a 6-hour exposure period for four consecutive days. The amount each rat received was determined from the body weight on each day of dosing. Two male rats were dosed with mineral oil and served as controls. All rats were observed for clinical signs and dermal effects on each day of dosing after the 6-hour exposure period.

The day after the last dose, approximately 0.6-1 mL of blood was collected into EDTA tubes from the orbital sinus of each rat. The rats were sacrificed by carbon dioxide asphyxiation. Plasma was prepared and stored frozen at approximately -20°C until analyzed for concentration o[redacted]

F. Assignment to Groups

The rats, selected on the bases of adequate body weight gain and freedom from any clinical signs of disease or injury, will be distributed by computerized, stratified randomization into study groups as designated in the Study Design, so that there are no statistically significant differences among group body weight means. The weight variation of selected rats will not exceed ± 20% of the mean weight.

After assignment to groups, each rat will be housed individually. Each rat will be assigned a unique 6-digit Haskell animal number and an individual cage identification number. Prior to assignment to groups, each rat will be temporarily identified by cage identification. After assignment to groups, the last 3 digits of the Haskell animal number will be tattooed on the tail of each rat. The Haskell animal number and cage identification number will both be included on the cage label.

At study start (test day 0) the rats will be 8 weeks old. On test day 0, when possible, rats with body weights that are not within ± 20% of the mean will be removed from study and replaced with rats having body weights within that range (subject to the same selection criteria as the original rats).

Rats that have not been assigned to a test group or which have been removed from study on test day 0, for out-of-range body weight, will be released for other laboratory purposes, or be sacrificed by carbon dioxide asphyxiation and discarded without pathological evaluation, at the discretion of the study director.

[redacted]

G. Administration of Test Substance

Approximately 24 hours prior to the first treatment, the fur of each rat will be closely shaved to expose the skin from the back and trunk. The target area to be treated is 5 cm x 7.4 cm = 37 cm^2. The 37 cm^2 treatment area is approximately equal to 10% of the total body surface area for rats in the 200 to 300 g range of body weights. The test substance will be moistened with mineral oil to form a thick paste and applied in a thin and uniform layer to cover as much surface of the target area as possible. For some doses, the volume of test substance may be too small to cover the entire target area. The approximate area to be covered will be marked on the back of each rat with a water insoluble marker and the percentage of the marked area covered will be documented in the study records. The test substance will be covered with a 2-ply gauze pad followed by successive layers of stretch gauze and self-adhesive bandage. The control rats will receive mineral oil at the same volume as the high-dose rats. The rats will be fitted with plastic collars during the exposure period to prevent ingestion of the test substance and disruption of the wrappings.

Groups of 10 male rats will be treated at dosages of 0 (control), 5, 50, or 500 mg of test substance per kg body weight. The rats will be treated for 28 consecutive days. The amount of test substance needed to treat each rat will be based on the most recently determined body weight measurement.

The exposure period will be approximately 6 hours. After the exposure period, the collars and wrappings will be removed and excess test substance will be washed off with soap and warm tap water. The test site of each rat will then gently be patted dry and the rat returned to its cage. Control animals will receive the same washing technique as the treated animals.

The animals will be reshaved during the study to facilitate the evaluation of dermal effects. The entire area that was originally shaved (including the untreated control skin) will be reshaved. The animals will be reshaved only after an evaluation.

H. Body Weights

The rats will be weighed at 3-4 day intervals during the study.

I. Food Consumption and Food Efficiency

The amount of food consumed by each rat will be determined weekly by weighing each feeder at the beginning and end of the week and subtracting the final weight and the amount of spillage from the feeder during the week from the initial weight. From these measurements, mean daily food consumption over the week will be determined. From the food consumption and body weight data, the mean daily food efficiency will be calculated.

[redacted]

J. Clinical Observations and Mortality

The rats will be observed for clinical signs and dermal effects after removal of the test substance. The rats will also be observed for clinical signs at each weighing. The Draize Scale will be used to score skin irritation. Adjacent areas of untreated skin will be used for comparison. Rats will be checked twice daily for mortality and for signs of illness, injury, or abnormal behavior.

K. Total Fluorine and Perfluorooctanoic Acid Level Evaluations

Blood (approximately 0.6-1 mL) will be collected into EDTA tubes from the orbital sinus of all rats on test days 0, 7, 14, 21, and 27. The blood will be collected from the animals approximately one hour after removal of the test substance. The blood will be refrigerated. The total fluorine content of the blood samples may be determined by using a Wickbold torch combustion method followed by analysis with a fluoride ion selective electrode. Initially, only the high-dose samples will be analyzed. Additional analyses will be requested by the study director based on the results of the high-dose analysis.

Additional blood will be collected from the vena cava of all rats at necropsy into a tube containing EDTA. Plasma will be prepared and stored frozen at -80°C to -20°C until analyzed for concentration of [redacted] Plasma will be evaluated from control and high-dose rats. The decision to evaluate low- and intermediate-dose groups will be based on results in the high-dose group.

Plasma samples will be extracted by organic solvent protein precipitation using a protein precipitation column, and then analyzed for [redacted] by LC-MS-MS.

L. Clinical Pathology Evaluation

A clinical pathology evaluation will be conducted on all rats the day after the last dose. The day before collection of blood samples for the clinical pathology evaluation, the animals will be placed in metabolism cages. These animals will be fasted overnight (approximately 16 hours) and urine will be collected from each animal. Blood samples for hematology and clinical chemistry measurements will be collected from the orbital sinus of each animal while the animal is under light carbon dioxide anesthesia. Blood samples for plasma fluoride and [redacted] will be collected at sacrifice from the abdominal *vena cava* of each animal while the animal is under carbon dioxide anesthesia. Additional blood collected from the *vena cava* will be placed in a serum tube, processed to serum, and frozen at -80°C to -20°C. Bone marrow smears will be prepared at the final sacrifice from all surviving animals and will be evaluated if warranted by experimental findings.

At the discretion of the study director or clinical pathologist, additional samples for selected clinical pathology tests will be collected from animals showing clinical evidence of toxicity or sacrificed *in extremis*.

[redacted]

1. Hematology

Blood samples will be evaluated for quality by visual examination prior to analysis.

The following hematology parameters will be determined:

red blood cell count
hemoglobin
hematocrit
mean corpuscular volume
mean corpuscular hemoglobin
mean corpuscular hemoglobin concentration
red cell distribution width
absolute reticulocyte count
platelet count
white blood cell count
differential white blood cell count
microscopic blood smear examination

prothrombin time
activated partial thromboplastin time

In addition, blood smears, stained with new methylene-blue, will be prepared from all hematology samples and will be evaluated, if required, to substantiate or clarify the results of hematology findings.

2. Clinical Chemistry

The following serum chemistry parameters will be determined:

aspartate aminotransferase
alanine aminotransferase
sorbitol dehydrogenase
alkaline phosphatase
total bilirubin
urea nitrogen
creatinine
cholesterol
triglycerides
glucose
total protein
albumin
globulin
calcium
inorganic phosphorus
sodium
potassium
chloride
fluoride[a]

3. Urinalysis

The following urinalysis parameters will be determined:

quality
color
clarity
volume
osmolality
pH
glucose
ketone
bilirubin
blood
urobilinogen
fluoride[b]
protein
microscopic urine sediment examination

a Fluoride determination will be analyzed from EDTA plasma.
b Fluoride determination will be analyzed from urine with EDTA added.

[redacted]

M. Anatomical Pathology

1. Pretest

See Materials and Methods, Section D. Pretest Period.

2. Dosing Phase

All rats found dead, accidentally killed, sacrificed *in extremis*, or sacrificed by design will undergo a gross and microscopic evaluation. All rats removed from study (except for out-of-range body weight on test day 0) will be sent to Pathology for gross evaluation and collection of tissues. Rats will be euthanatized by carbon dioxide anesthesia and exsanguination. Rats sacrificed by design will be fasted after 3 p.m. on the afternoon before their scheduled sacrifice. A final sacrifice will be performed on surviving rats following the final clinical pathology evaluation. The order of sacrifice for scheduled deaths will be random among all treatment groups.

Blood samples for coagulation parameters and possible future analysis will be collected at sacrifice from the abdominal *vena cava* while the rat is under carbon dioxide anesthesia. Plasma will be prepared from the samples reserved for possible analysis, and the plasma will be stored at approximately -80°C to -20°C (see Section K.).

[redacted]

The following tissues will be collected from rats which are found dead or accidentally killed (tissue integrity permitting), sacrificed *in extremis*, removed from study (except out-of-range body weight on test day 0), or sacrificed by design.

Digestive System
- liver
- esophagus
- stomach
- duodenum
- jejunum
- ileum
- cecum
- colon
- rectum
- salivary glands
- pancreas

Urinary System
- kidneys
- urinary bladder

Respiratory System
- lungs
- trachea
- nasal tissue
- larynx
- pharynx

Cardiovascular System
- heart
- aorta

Hematopoietic System
- spleen
- thymus
- mandibular lymph node
- mesenteric lymph node
- bone marrow[a]

Endocrine System
- pituitary gland
- thyroid gland
- parathyroid glands
- adrenal glands

Nervous System
- brain (cerebral cortex, cerebellar cortex, medulla/pons)
- spinal cord (cervical, mid-thoracic, lumber)
- sciatic nerve

Musculoskeletal System
- skeletal muscle
- femur/knee joint
- sternum

Reproductive System
Male
- testes
- epididymides
- prostate
- seminal vesicles

Miscellaneous
- skin
- eyes (including optic nerve)
- gross observations[b]

a Bone marrow will be collected with the femur and sternum.
b Gross observations made at necropsy for which histopathology is not appropriate (e.g., fluid, ruffled fur, and missing anatomic parts) will generally not be collected.

All tissues will be placed in the appropriate fixative.

Rats sacrificed by design will have the following organs weighed: liver, kidneys, adrenal glands, thymus, brain, spleen, heart, testes, and epididymides. Relative organ weights (percent of final body weight; ratio to brain weight) will be calculated. Final body weights determined just prior to necropsy will be used in the assessment of organ weight changes. Organs from rats found dead, sacrificed *in extremis*, or accidentally killed may be weighed at the discretion of the pathologist or study director.

Bone marrow smears will be prepared at the final sacrifice from all surviving rats and will be evaluated if warranted by experimental findings.

All tissues collected from rats in the high-dosage and control groups, and from rats that are found dead or accidentally killed (tissue integrity permitting), or are sacrificed *in extremis*, will be further processed to slides, stained with hematoxylin and eosin, and examined microscopically.

[redacted]

Most gross lesions and any additional target organs from rats in the low- and intermediate-dosage groups will be evaluated microscopically. Selected gross observations for which a microscopic diagnosis would not be additive (e.g., osteoarthritis, pododermatitis, chronic tail dermatitis, calculus, and deformities of the teeth, toe, tail, or ear pinna) will be saved, but will generally not be processed for microscopic evaluation. Tissues from rats removed from study (for other than out-of-range body weight on test day 0), will not be processed for microscopic evaluation unless considered necessary by the study director or pathologist.

Additional procedures to identify and/or clarify histologic features of lesions may be performed at the discretion of the pathologist and will be documented in the final report.

[redacted]

STATISTICAL ANALYSES

Significance will be judged at $p < 0.05$.

Parameter	Preliminary Test	Method of Statistical Analysis: If preliminary test is not significant	Method of Statistical Analysis: If preliminary test is significant
Body Weight Body Weight Gain Food Consumption Food Efficiency Organ Weight	Test for lack of trend[5]	Sequential application[6] of the Jonckheere-Terpstra trend test[7]	Preliminary tests for pairwise comparison
	OR[a]		
	Levene's test for homogeneity[8] and Shapiro-Wilk test[9] for normality[b]	One-way analysis of variance[10] followed with Dunnett's test[11]	Kruskal-Wallis test[12] followed with Dunn's test[13]
Clinical Pathology[c]	Levene's test for homogeneity[8] and Shapiro-Wilk test[9] for normality[b]	One-way analysis of variance[10] followed with Dunnett's test[11]	Kruskal-Wallis test[12] followed with Dunn's test[13]
Survival Incidence of Clinical Observations Incidence of Dermal Effects	None	Cochran-Armitage test for trend[10][d]	

a Pairwise comparisons and associated preliminary tests are only conducted if the test for lack of trend is significant.
b If the Shapiro-Wilk test is not significant but Levene's test is significant, a robust version of Dunnett's test will be used.
c When an individual observation is recorded as being less than a certain value, calculations are performed on half the recorded value. For example, if bilirubin is reported as <0.1, 0.05 is used for any calculations performed with that bilirubin data.
d If the incidence is not significant, but a significant lack of fit occurs, then Fisher's Exact test[10] with a Bonferroni correction is used.

Other methods will be used, if appropriate, at the time of analysis. The statistical methods used will be described in the final report.

SAFETY AND HOUSEKEEPING

Good housekeeping procedures will be practiced to avoid contamination of the test substance and to avoid potential health hazards. To avoid skin contact, gloves will be worn when handling the test substance. Animal carcasses and feces will be incinerated.

[redacted]

RECORDS AND SAMPLE STORAGE

All data and records for analytical characterizations conducted by the sponsor will be archived by the sponsor.

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. EPA/TSCA Good Laboratory Practice Standards (40 CFR 792). (1989).
2. OECD Principles of Good Laboratory Practice (as revised in 1997, published in ENV/MC/CHEM(98)17 (OCDE/GD(92)32).
3. United States Environmental Protection Agency (EPA), Office of Prevention, Pesticides, and Toxic Substances (OPPTS) Health Effects Test Guidelines, OPPTS 870.3200, 21/28-Day Dermal Toxicity (AUG-1998).
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7. Jonckheere, A.R. (1954). A distribution-free K-sample test against ordered alternatives. *Biometrika* 41, 133-145.
8. Levene, H. (1960). Robust test for equality of variances. *Contributions to Probability and Statistics* (I. Olkin, ed.), pp 278-292. Stanford University Press, Palo Alto.
9. Shapiro, S.S. and Wilk., M.B. (1965). An analysis of variance test for normality (complete samples). *Biometrika* 52, 591-611.
10. Snedecor, G.W. and Cochran, W.G. (1967). *Statistical Methods*, 6th edition, pp 246-248 and 349-352. The Iowa State University Press, Iowa.
11. Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Amer. Statist. Assoc.* 50, 1096-1121.
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14. Fisher, R.A. (1985). *Statistical Methods for Research Workers*, 13th edition. Haffner, New York.

[redacted]

PROTOCOL APPENDIX

Study Function	Study Personnel
Study Director	Carol Finlay Staff Toxicologist
Clinical Pathology Evaluation	Nancy E. Everds Principal Research Clinical Pathologist and Manager
Perfluorooctanoic Acid Level Evaluations	Shawn A. Gannon Senior Staff Toxicologist
Anatomic Pathology Evaluation	John F. Hansen Principal Research Pathologist

Study Dates	
Initiation of Test Substance Administration	November 25, 2002
Collection of Blood for Fluorine Analysis	November 25, 2002 December 2, 2002 December 9, 2002 December 16, 2002 December 22, 2002
Clinical Pathology Evaluations	December 23, 2002
Anatomic Pathology Evaluation	December 23, 2002
Scheduled Sacrifice	December 23, 2002

[redacted]

SIGNATURES

Approved by: _____ Nov 28 2002
Jamie A. Britton
Primary Technician
Date

_____ 22-Nov-2002
Nancy E. Evans
Clinical Pathologist
Date

_____ 22-Nov-2002
John F. Hansen
Anatomic Pathologist
Date

_____ 22-Nov-2002
Scott E. Loveless
Research Manager and Director
Date

_____ 22-Nov-2002
(Carol Finley
Study Director
Date

cc: [redacted]

[redacted]

Work Request Number 13951
Service Code 1012

Protocol Amendment No. 1

The protocol is amended as follows (changes bolded):

1. Page 4, Study Design, add test day 21 to Frequency for Blood Collection for Total Fluorine Analysis.

2. Page 8, Section L. Clinical Pathology Evaluation, replace the 5th sentence with "Blood samples for coagulation, plasma fluoride, and PFOA will be collected at sacrifice from the abdominal *vena cava* of each animal while the animal is under carbon dioxide anesthesia."

Rationale: These items were inadvertently omitted from the original protocol.

Approved by: Carol Finlay 27 Mar 03
Carol Finlay
Study Director
Date

cc: [redacted]

[redacted]

[redacted]

Protocol Amendment 2

The protocol is amended as follows:

1. Assign Anatomic Pathologist responsibilities to Peter C. Mann, Veterinary Pathologist.

Rationale: Study personnel reassigned due to organizational change.

Approved by: Carol Finley 30-Jan-2003
Carol Finley, Study Director — Date

Peter C. 01 Feb 2003
Peter C. Mann, Anatomic Pathologist — Date

cc: [redacted]

[redacted]

[redacted]

Protocol Amendment 3

The protocol is amended as follows:

Page 8, section K. Total Fluorine and [redacted]ovel Evaluations, change: the second to last sentence in the first paragraph from "Initially, only the high-dose samples will be analyzed." to: "Initially, only the day 27 samples from the first five rats of each group will be analyzed."

Reason: The fluorine levels may be compared to fluorine levels for other studies conducted with this test substance.

Approved by: Carol Kinkley 9.5.03
Carol Kinkley, Study Director — Date

cc: [redacted]

[redacted]

JUL. 23. 2003 6:21AM HASKELL 1 / 1300 NO. 6286 P. 21/23

H-25509: Repeated-Dose Dermal Toxicity
28-Day Study in Male Rats

DuPont-11763

[redacted]

Protocol Amendment 4

The protocol is amended as follows:

Protocol Amendment 5, add the following sentence: "Fluorine blood Analysis will be perfomed at Jackson Laboratory, Deepwater, N.J. 08023".

Reason: To add the name and address of the fluorine blood analysis.

Approved by: Carol Ashley, Study Director 25-March-2003 Date

cc: [redacted]

-1-

RECEIVED TIME JUL.23. 6:26AM PRINT TIME JUL.23. 6:38AM

Exygen Research

Page 47 of 72

[redacted]

[redacted]

Protocol Amendment 5

The protocol is amended as follows:

1. Protocol Amendment 4, delete the item.

   Reason: The item is deleted because the sentence needs to be added to the protocol rather than to the protocol amendment. This change also addresses the incorrect protocol amendment number.

2. Protocol, Page 8, Section K. Fluorine Blood Analysis and [redacted] Level Evaluations add the following sentence: "Fluorine blood analysis will be performed at Jackson Laboratory, Deepwater, N.J. 08023".

   Reason: To add the name and address of the laboratory performing the fluorine blood analysis.

Approved by: [redacted] Chief Pathology Study Director — 14 March 2003 Date

cc: [redacted]

[redacted]

JUL. 23. 2003 6:22AM HASKELL 1 / 1300 NO. 6286 P. 23/23

[redacted]

Protocol Amendment 6

The protocol is amended as follows:

The results of the [redacted] plasma analysis will be presented in a supplemental report.

Reason: The [redacted] plasma analysis method has not been fully validated.

Approved by: Carol Hinsley 25-May-2003
Carol Hinsley
Study Director
Date

cc: [redacted]

-1-

RECEIVED TIME JUL.23. 6:26AM PRINT TIME JUL.23. 6:38AM

[redacted]

H-25509: Repeated-Dose Dermal Toxicity
28-Day Study in Male Rats

DuPont-11763

[redacted]

Protocol Amendment 7

The protocol is amended as follows:

Page 8, Materials and Methods, Section K. Total Fluorine and [redacted] Level Evaluations, third paragraph, change to read: "Plasma samples will be extracted by organic solvent protein precipitation and then analyzed for [redacted] by an appropriate method at Exygen Research (3058 Research Drive, State College, PA 16801)."

Reason: To add the name and address of the laboratory performing the [redacted] analysis.

Approved by: Carol Shirley, Study Director — 25-July-2003, Date

cc: [redacted]

[redacted]

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Page 1 of 1

PROTOCOL DEVIATION

Deviation Number: 1
Date of Occurrence: 03/17/03, 07/22/03

[redacted] Protocol Number: DuPont-11763

DESCRIPTION OF DEVIATION

1. Section 12. Analytical Method Section 3.5.1–did not prepare 100 µg/mL stock of internal standard, used alternate volumes for fortification preparation, and prepared additional concentrations.
2. Section 12. Analytical Method Section 4.3.a–did not prepare a reagent blank with Set 072203A.

ACTIONS TAKEN
for example - deviation issued; SOP revision, etc.

1. Protocol Deviation issued and will revise the method to account for variable concentrations and preparations of standards as long as the concentrations are accurately recorded.

2. Protocol Deviation issued.

Recorded By: Emily R Decker Date: 8/14/03

IMPACT ON STUDY

1. No negative impact because standard preparation was accurately documented and the actual concentrations prepared were used for calculation purposes.

2. No negative impact because all other matrix controls and QC samples meet the acceptance criteria for that set.

Emily R Decker — Principal Investigator Signature — Date: 8/18/03

Study Director Signature — Date: 8/22/03

S. Mann — S. MARK KENNEDY — Management Signature — Date: 8/22/03

(Sponsor) N/A — Date: N/A

Exygen QAU Review: LJS 08/14/03

U:\Forms\PROTOCOL_DEVIATION.doc 9/26/2002/5

3058 Research Drive
State College, PA 16801, USA
T: 800.281.3219
F: 814.272.1019
exygen.com

[redacted]

APPENDIX B

Analytical Method [redacted] "Method of Analysis for the Determination of [redacted] in Serum by LC/MS/MS"

[redacted]

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TITLE

Method of Analysis for the Determination [redacted] in Serum by LC/MS/MS

AUTHOR

Emily Decker

DATE ISSUED

June 9, 2003

SPONSOR

DuPont
Haskell Laboratory for Health & Environmental Sciences
P.O. Box 50
Newark, DE 19714-0050 USA
DuPont Study Number: DuPont-13090

PERFORMING LABORATORY

Exygen Research
3058 Research Drive
State College, PA 16801

METHOD NUMBER

[redacted]

TOTAL NUMBER OF PAGES

20

3058 Research Drive
State College, PA 16801, USA
T: 800.281.3219
F: 814.272.1019
exygen.com

Exygen Study No.: 008-317

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TITLE

Method of Analysis for the Determination of Perfluorooctanoic Acid (PFOA) in Serum by LC/MS/MS

AUTHOR

Emily Decker

DATE ISSUED

June 9, 2003

SPONSOR

DuPont
Haskell Laboratory for Health & Environmental Sciences
P.O. Box 50
Newark, DE 19714-0050 USA
DuPont Study Number: DuPont-13090

PERFORMING LABORATORY

Exygen Research
3058 Research Drive
State College, PA 16801

METHOD NUMBER

ExM-008-276

TOTAL NUMBER OF PAGES

20

3058 Research Drive
State College, PA 16801, USA
T: 800.281.3219
F: 814.272.1019
exygen.com

[redacted]

MANAGEMENT APPROVAL

Emily R. Decker 6/9/03
Emily R. Decker — Date
Scientist
Exygen Research

6/9/03
John Flaherty — Date
Vice President
Exygen Research

10 JUNE 2003
S. Mark Kennedy — Date
Sponsor Representative
DuPont

[redacted]

TABLE OF CONTENTS

[redacted]

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[redacted]

1. SUMMARY

This report details a method of analysis for [redacted] in serum.

PFOA is extracted from serum by protein precipitation in acetonitrile. Quantification of [redacted] is accomplished by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis using selected reaction monitoring (SRM). The chemical formula of [redacted] is given in section 2 of this method.

The lower limit of quantitation (LLOQ) for this method is 10 ppb for [redacted] in serum.

Quantification is performed using extracted calibration standards containing an internal standard.

This method was developed using monkey serum. The overall percent recovery ± standard deviation for [redacted] in monkey serum at 10, 100, and 1000 ppb was 94% ± 6.9% (Table I).

A representative calibration curve for [redacted] monkey serum is shown in Figure 1. Representative chromatograms for [redacted] in monkey serum are shown in Figures 2 to 4.

[redacted]

2. EXPERIMENTAL COMPOUNDS

The structure of [redacted] and the internal standard are given below.

[redacted]

[redacted]

3. CHEMICALS AND SUPPLIES

3.1. CHEMICALS

Chemical	Grade	Source	Catalog No.
Methanol (MeOH)	HPLC	EM Science	JT9093-2
Acetonitrile (ACN)	HPLC	EM Science	AX0145-1
Ammonium Acetate	Reagent	Sigma-Aldrich	A-7330
OmniSolv Water	HPLC	EM Science	WX0004-1

3.2. STANDARDS

Standard	Lot Number	Purity (%)	Source
[redacted]			Oakwood Products DuPont

[redacted]

3.3. Equipment and Supplies

Equipment	Supplier
Balance, analytical (display at least 0.0001 g)	Mettler
Centrifuge	Eppendorf
2 mL eppendorf centrifuge tubes	VWR
Disposable micropipets (50-100uL, 100-200uL)	Drummond (VWR)
Class A pipets and volumetric flasks	various suppliers
Hypercarb drop-in guard column (4 mm) (part # 844017-400)	Keystone
Stand-alone drop-in guard cartridge holder	Keystone
125-mL LDPE narrow-mouth bottles	Nalgene
2 mL clear HPLC vial kit (cat # 5181-3400)	HP
Standard lab equipment (graduated cylinders, disposable tubes etc.)	various suppliers
LC/MS/MS and HPLC systems	As described in section 4.5.

Note: Equivalent materials may be substituted for those specified in this method if they can be shown to produce satisfactory results.

Notes:

1. In order to avoid contamination, the use of disposable labware is highly recommended (tubes, pipets, etc.).
2. PTFE or PTFE lined containers or equipment, including PTFE-lined HPLC vials for the HPLC autosampler must not be used.
3. It is necessary to check the solvents (methanol) for the presence of contaminants by LC/MS/MS before use. Certain lot numbers have been found to be unsuitable for use.
4. Use disposable micropipets or pipets to aliquot standard solutions to make calibration standards and sample fortifications.
5. The hypercarb cartridges should be changed when the system contaminants (elevated baseline) move to within 1 minute from the elution o[redacted]

3.4. Solutions

(1) 2 mM ammonium acetate solution is prepared by weighing 0.15 g of ammonium acetate and dissolving in 1 L of OmniSolv Water.

Note: The aforementioned example is provided for guidance, alternative volumes may be prepared as long as the ratios of the solvent to solute are maintained.

[redacted]

3.5. Preparation of Standards and Fortification Solutions

Analytical standards are used for three purposes:

1. Calibration Standards – These standards are prepared in control monkey serum and are used to calibrate the response of the detector used in the analysis.
2. Laboratory Control Spikes – These fortifications are usually prepared at concentrations corresponding to the LLOQ and 10x LLOQ and are used to determine analytical recovery. Laboratory control spikes are prepared in control monkey serum.
3. Matrix Spikes – These fortifications are prepared by spiking into the field samples at known concentrations. Matrix spikes are used to evaluate the effect of the sample matrix on analytical recovery.

The absolute volumes of the standards may be varied by the analyst as long as the correct proportions of solute to solvent are maintained.

3.5.1. Stock solution

Prepare individual stock solutions of 100 µg/mL of [redacted] and ^{13}C-[redacted] by weighing out 10 mg of analytical standard (corrected for purity) and dilute to 100 mL with methanol in a 100-mL volumetric flask. Each stock solution (in 125-mL LDPE bottles) is to be stored in a refrigerator at 2°C to 6°C and is stable for a maximum period of 1 year from the date of preparation.

3.5.2. Fortification Solutions

a. Prepare a fortification standard of 10.0 µg/mL for [redacted] by diluting 10.0 mL of the [redacted] solution described in 3.5.1 to 100 mL with acetonitrile in a volumetric flask.
b. Prepare a fortification standard of 1.0 µg/mL for ^{13}C-[redacted] by diluting 1.0 mL of the ^{13}C-[redacted] solution described in 3.5.1 to 100 mL with acetonitrile in a volumetric flask.
c. Prepare a fortification standard of 1.0 µg/mL for [redacted] containing 0.005 µg/mL of ^{13}C-[redacted] by diluting 10.0 mL of the [redacted] solution described in 3.5.2.a and 0.5 mL of the ^{13}C-[redacted] solution described in 3.5.2.b to 100 mL with acetonitrile in a volumetric flask.
d. Prepare a fortification standard of 0.1 µg/mL for [redacted] containing 0.005 µg/mL of ^{13}C-[redacted] by diluting 1.0 mL of the [redacted] solution described in 3.5.2.a and 0.5 mL of the ^{13}C-[redacted] solution described in 3.5.2.b to 100 mL with acetonitrile in a volumetric flask.
e. Prepare a fortification standard of 0.01 µg/mL for [redacted] containing 0.005 µg/mL of ^{13}C-[redacted] by diluting 0.1 mL of the [redacted] solution described in 3.5.2.a and 0.5 mL of the ^{13}C-[redacted] solution described in 3.5.2.b to 100 mL with acetonitrile in a volumetric flask.

[redacted]

Store all fortification standard solutions in a refrigerator (in 125-mL LDPR bottles) at 2°C to 6°C for a maximum period of 1 year from the date of preparation, after which time it is necessary to make new standards using the stock solution.

3.5.3. Calibration Standards

The calibration standards are processed through the extraction procedure, identical to the samples. The fortification of the standards before extraction is done according to the following table:

Conc. Of Mixed Fortification Solution (µg/mL)	Fortification Volume (µL)	Volume of Control Sample (mL)	Conc. of Extracted Calibration Standard (ppb)
0.01	100	0.1	10
0.01	200	0.1	20
0.1	50	0.1	50
0.1	100	0.1	100
0.1	200	0.1	200

4. METHOD

4.1. FLOW DIAGRAM

The flow diagram of the method is given below, followed by a detailed description of each step.

Method Flow Diagram

Measure 0.1 mL of sample (using disposable micropipette)
(fortify samples when designated)
↓
Protein precipitation in acetonitrile
↓
LC/MS/MS analysis

4.2. SAMPLE PROCESSING

No sample processing is needed for serum samples. However, frozen samples must be allowed to completely thaw, un-aided, at room temperature. Samples stored refrigerated should also be allowed to equilibrate to room temperature. All samples must be thoroughly mixed before being sampled for extraction.

[redacted]

4.3. Sample Preparation

a. Each batch of samples extracted (typically 30 or less) must include at least one reagent control (acetonitrile blank), one matrix control (method blank), and two matrix controls fortified at known concentrations to verify procedural recovery for the batch.
b. At least one sample per batch should be extracted in duplicate
c. At least one sample extracted should be separately fortified at a known concentration and carried through the procedure to verify recovery. Additional matrix spikes may be performed at the sponsor's request.

4.4. Extraction

1. Measure 0.1 mL of sample into 2 mL eppendorf centrifuge tubes (fortify with analyte as needed, close lid, and vortex ~ 10 seconds).
2. Add enough acetonitrile containing internal standard at 0.005 µg/mL (accounting for fortification volume) to make extraction volume 500 µL and vortex for ~ 10 seconds.
3. Centrifuge for ~ 10 minutes at ~ 14,000 rpm.
4. Analyze samples using electrospray LC/MS/MS.

4.5. Quantitation

4.5.1. LC/MS/MS System and Operating Conditions

Instrument:	PE SCIEX API 4000 Biomolecular Mass Analyzer SCIEX Turbo Ion Spray Liquid Introduction Interface
Computer:	Dell OptiPlex GX 110
Software:	PE Sciex Analyst 1.2
HPLC Equipment:	Hewlett Packard (HP) Series 1100 Quatpump G1311A Vacuum Degasser G1322A Autoinjector G1313A Column Compartment G1316A

Note: Two 4 × 10 mm hypercarb drop-in guard cartridges (Keystone, part # 844017-400) are attached in-line after the purge valve and before the sample injector port to trap any residue contaminants that may be in the mobile phase and/or HPLC system.

HPLC Column: Genesis C_8 (Jones Chromatography), 2.1 mm x 50 mm, 4µ

[redacted]

Column Temperature: 30° C
Injection Volume: 5 µL
Mobile Phase (A): 2 mM Ammonium Acetate in OmniSolv Water
Mobile Phase (B): Methanol

Time	%A	%B	Flow (mL/min)
0.0	40	60	0.3
3	40	60	0.3
3.5	0	100	0.3
3.7	0	100	0.5
7	0	100	0.5
7.5	40	60	0.5
9	40	60	0.5
9.5	40	60	0.3
12	40	60	0.3

It may be necessary to adjust the HPLC gradient in order to optimize instrument performance.

Ions monitored:

Analyte	Mode	Transition Monitored	Approximate Retention Time
[redacted]	Negative	413 → 369	~2.8 min.
	Negative	415 → 370	~2.8 min.

The retention times may vary, on a day-to-day basis, depending on the batch of mobile phase etc. Drift in retention times is acceptable within an analytical run, as long as the drift continues through the entire analysis and the standards are interspersed throughout the analytical run.

Note: An alternative LC/MS/MS system may be used once demonstrated to be equivalent.

4.5.2. Tune File Parameters

The mass spectrometer is tuned for the analyte by infusing a ~ 1 µg/mL standard solution of [redacted] (at 10 µL/min, using an infusion pump) via a "T" into a stream of mobile phase containing 60% methanol and 40% 2mM ammonium acetate at 0.3 mL/min flow rate. The analyte is initially tuned for the parent ion and then tuned for the product ion. Once the instrument is tuned, the optimized parameters are saved as a tune file. This tune file is then used during routine analysis.

4.5.3. Calibration Procedures

a. Inject the same aliquot (5 µL) of each calibration standard into the LC/MS/MS.

[redacted]

b. Use linear standard curves for quantitation. Linear standard curves are generated for each analyte by linear regression using 1/x weighting of the ratio analyte peak area/internal standard peak area versus the ratio of the concentration of analyte/concentration of internal standard using Analyst 1.2 (or equivalent) software system. Any calibration standard found to be a statistical outlier by using the appropriate outlier test (e.g. Huge Outlier Test), may be excluded from the calibration curve. However, the total number of calibration standards that could be excluded must not exceed 20% of the total number of standards injected and at least one calibration standard at the LLOQ must be retained.

c. The correlation coefficient (r) for calibration curves generated must be ≥0.9925 (r^2 ≥0.985). If calibration results fall outside these limits, then appropriate steps must be taken to adjust instrument operation, and the standards or the relevant set of samples should be reanalyzed.

4.5.4. Sample Analysis

a. Inject the same aliquot (5 µL) of each standard, sample, recovery, control, etc. into the LC/MS/MS system.

b. Standards corresponding to at least five or more concentration levels must be included in an analytical set.

c. An entire set of calibration standards should be injected at the beginning of a set followed by calibration standards interspersed every 5-10 samples (to account for a second set of standards). As an alternative, an entire set of calibration standards may be included at the beginning and at the end of a sample set. In either case, calibration standards must be the first and last injection in a sample set.

d. The concentration of each sample/fortification/control is determined from the standard curve, based on the peak area of each analyte. The standard responses should bracket responses of the residue found in each sample set. If necessary, dilute the samples in acetonitrile to give a response within the standard curve range.

e. Fortification recoveries falling within 85 to 115% (80 to 120% for levels at the LLOQ) are considered acceptable.

f. Extracted samples must be stored refrigerated between 2°C to 6°C until analysis.

g. Samples in which no peaks are detected (i.e. signal : noise ratio < 3:1) at the corresponding analyte retention times will be reported as ND (not detected). Samples in which peaks are detected at the corresponding analyte retention

[redacted]

times but are less than the lowest concentration of the calibration standards (10 ppb where LLOQ = 10 ppb) will be reported as <10 ppb.

4.6. ACCEPTANCE CRITERIA

The following criteria must be met to ensure the presence of [redacted] and ^{13}C-[redacted]

1. The chromatograms must show the following peaks:
   [redacted]: a daughter ion at 369 amu from a parent of 413 amu
   ^{13}C[redacted]: a daughter ion at 370 amu from a parent of 415 amu

2. Any analyte present in method blanks must be at least 5-fold lower than the LLOQ. Any analyte present in the reagent blank must be at least 5-fold lower than the LLOQ.

3. Recoveries of lab control spikes and matrix spikes must be between 85-115% (80-120% for levels at the LLOQ) of their known values. Any method fortification (lab control spike) falling outside the acceptable limits warrants re-extraction of the entire analytical set. Any matrix spike outside the acceptable range should be evaluated by the analyst to determine if re-extraction is warranted.

4. Any calibration standard found to be a statistical outlier by using an appropriate outlier test, may be excluded from the curve. However, the total number of calibration standards that could be excluded must not exceed 20% of the total number of standards injected and at least one calibration standard at the LLOQ must be retained.

5. The correlation coefficient (r) for calibration curves generated must be ≥0.9925 (r^2 ≥0.985). If calibration results fall outside these limits, then appropriate steps must be taken to adjust instrument operation, and the standards or the relevant set of samples should be reanalyzed.

4.7. PERFORMANCE CRITERIA

The following two criteria must be performed as a system suitability test, before the commencement of analysis when using an instrumentation set-up that has not been used for this method.

First Criterion:
Run a standard solution on LC/MS/MS corresponding to the estimated LLOQ and obtain a signal-to-noise ratio of at least 9:1, compared to a reagent blank. If this criterion cannot be met, optimize and change instrument operating parameters.

[redacted]

Second Criterion:
Run a set of standards of five or more concentration levels, spanning a range starting at or below the LLOQ up to the highest concentration level to be included in the analysis. Generate a calibration curve for each analyte and obtain a linear regression with a coefficient of determination (r^2) of at least 0.985. Once this criterion is met, samples may be analyzed with standards interspersed.

4.8. Time Required for Analysis

A set of 35 samples (1 reagent control, 1 matrix control, 1 laboratory spike, 2 laboratory control spikes, and 30 samples) can be taken through the extraction procedure in approximately 8 hours by one person. The LC/MS/MS analysis (standards and 35 samples) will take approximately 9 hours.

5. CALCULATIONS

a. Use Equation 1 to calculate the amount of analyte found (in ng/mL, based on peak area) using the standard curve (linear regression parameters) generated by the Mass Lynx software program.

Equation 1:
Analyte found (ppb) = $\frac{(\text{Analyte Peak area/IS peak area}) - \text{intercept})}{\text{slope}} \times \text{IS conc. (ng/mL)}$

b. For samples fortified with known amounts of analyte prior to extraction, use Equation 2 to calculate the percent recovery.

Equation 2:
Recovery (%) =

$$\frac{\text{Analyte found (ppb)} - \text{avg. Analyte found in control (ppb)}}{\text{Amount Analyte added (ppb)}} \times 100\%$$

6. SAFETY

The analyst should read the material safety data sheets for all standards and reagents before performing this method. Use universal precautions when handling standards and reagents, including working in fume hoods and wearing laboratory coats, safety glasses, and gloves. Use blood-borne pathogen handling precautions when handling serum.

[redacted]

Table I. Recovery of [redacted] from Fortifications In Monkey Serum

Summary of [redacted] Recoveries for 10 ppb Fortification in Monkey Serum

Exygen ID	Sponsor ID	Extraction Date	Analysis Date	Fort. Level (ppb)	% Recovery
0300808 Spk A	Lot 40K7400	05/30/03	05/30/03	10	95
0300808 Spk B	Lot 40K7400	05/30/03	05/30/03	10	94
0300808 Spk C	Lot 40K7400	05/30/03	05/30/03	10	98
				AVERAGE:	96
				STANDARD DEVIATION:	2.1
				RELATIVE STANDARD DEVIATION:	2.2

Summary of [redacted] Recoveries for 100 ppb Fortification in Monkey Serum

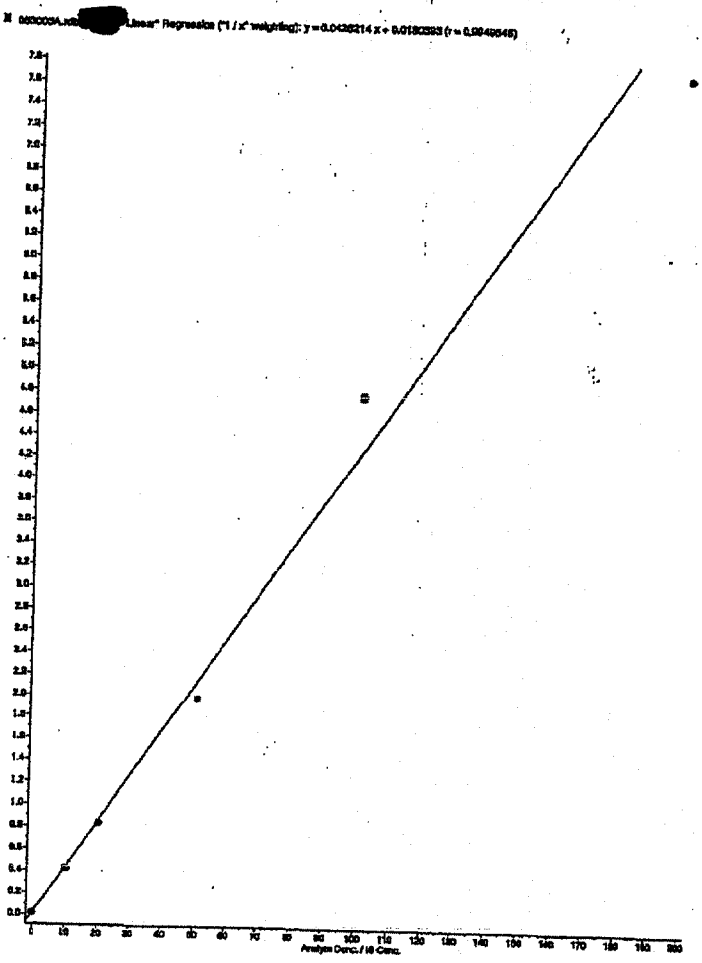
Exygen ID	Sponsor ID	Extraction Date	Analysis Date	Fort. Level (ppb)	% Recovery
0300808 Spk D	Lot 40K7400	05/30/03	05/30/03	100	92
0300808 Spk E	Lot 40K7400	05/30/03	05/30/03	100	90
0300808 Spk F	Lot 40K7400	05/30/03	05/30/03	100	93
				AVERAGE:	92
				STANDARD DEVIATION:	1.5
				RELATIVE STANDARD DEVIATION:	1.7

Summary of [redacted] Recoveries for 1000 ppb Fortification in Monkey Serum

Exygen ID	Sponsor ID	Extraction Date	Analysis Date	Fort. Level (ppb)	% Recovery
0300808 Spk G	Lot 40K7400	05/30/03	05/30/03	1000	107
0300808 Spk H	Lot 40K7400	05/30/03	05/30/03	1000	95
0300808 Spk I	Lot 40K7400	05/30/03	05/30/03	1000	81
				AVERAGE:	94
				STANDARD DEVIATION:	13.0
				RELATIVE STANDARD DEVIATION:	13.8
				OVERALL AVERAGE:	94
				OVERALL STANDARD DEVIATION:	6.9
				OVERALL RELATIVE STANDARD DEVIATION:	7.3

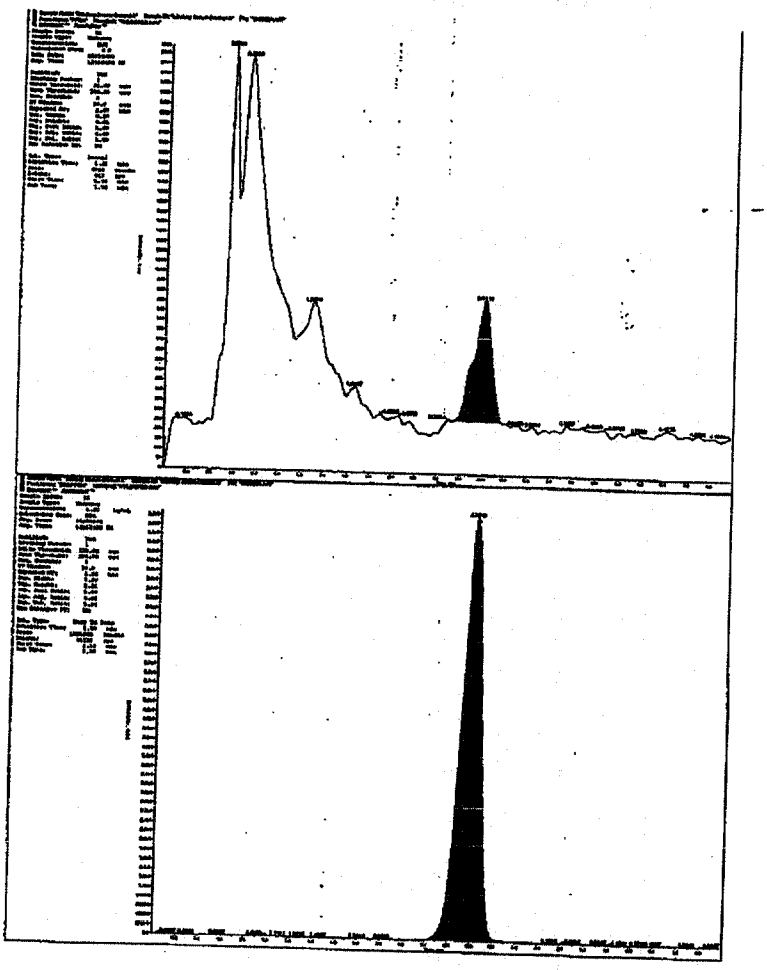
[redacted]

Figure 1. Calibration curve for [redacted] in Control Monkey Serum



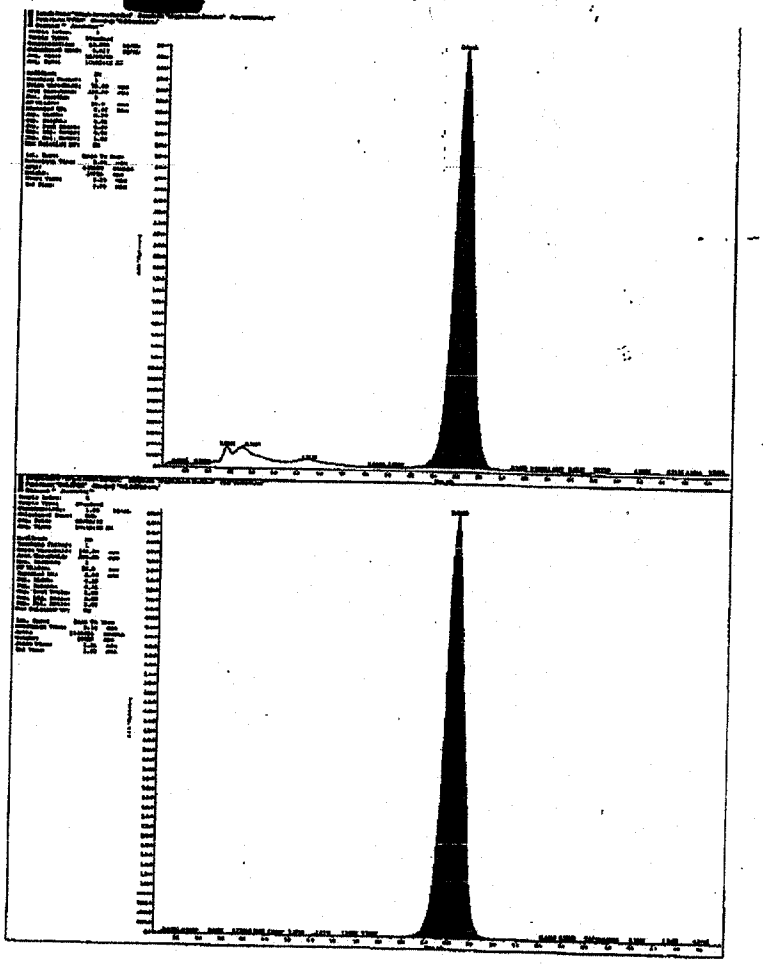
[redacted]

Figure 2. Representative Chromatogram of a Control Monkey Serum Sample for [redacted] with ~ 5 ng/mL of ^{13}C-[redacted]



[redacted]

Figure 3. Representative Chromatogram of a 10 ppb Standard for [redacted] in Control Monkey Serum with ~ 5 ng/mL of ^{13}C-[redacted]



[redacted]

Figure 4. Representative Chromatogram of Control Monkey Serum Fortified at 10 ppb with [redacted] and with ~ 5 ng/mL of ^{13}C-[redacted]