COVANCE
THE DEVELOPMENT SERVICES COMPANY

Sponsor:

3M
St. Paul, Minnesota

PROTOCOL

Study Title:

104-Week Dietary Chronic Toxicity and Carcinogenicity Study with Perfluorooctane Sulfonic Acid Potassium Salt (PFOS; T-6295) in Rats

Date:

April 15, 1998

Performing Laboratory:

Covance Laboratories Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Study Identification:

Proposal No. 5353

Covance 6329-183

Study
104-Week Dietary Chronic Toxicity and Carcinogenicity Study with Perfluorooctane Sulfonic Acid Potassium Salt (PFOS; T-6295) in Rats

Purpose
To assess the chronic toxicity and carcinogenicity of the test material when administered in the diet to rats for at least 104 weeks

Sponsor
3M
Toxicology Services
Building 220-2E-02, 3M Center
St. Paul, Minnesota 55144-1000

Study Monitor
Andrew M. Seacat, PhD
3M
Phone No.: 612.575.3161
Facsimile No.: 612.733.1773

Study Location
Covance Laboratories Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Mailing Address: PO Box 7545
Madison, Wisconsin 53707

Study Director
Peter J. Thomford, PhD
Covance Laboratories Inc.
Phone No.: 608.241.7207
Facsimile No.: 608.242.2736

Toxicologist
Thomas E. Ryan, BS
Covance Laboratories Inc.

Proposed Study Timetable
In-Life Start Date: April 20, 1998
In-Life End Date: April 24, 2000
Audited Draft Report Date: To be determined

Regulatory Compliance
The study will be conducted in compliance with the Good Laboratory Practice Regulations as set forth in Title 40 of the US Code of Federal Regulations Part 792, issued November 29, 1983 (effective December 29, 1983), and with any applicable amendments.

Animal Care and Use Statement
All procedures in this protocol are in compliance with the Animal Welfare Act Regulations, 9 CFR 1-4. In the opinion of the Sponsor and study director, the study does not unnecessarily duplicate any previous work.

Quality Assurance
The protocol, study conduct, and final report will be audited by the Covance Quality Assurance Unit (QAU). The proliferation cell nuclear antigen evaluation, data, and report will be audited by the QAU of Pathology Associates International. Liver and serum analyses, data, and report will be audited by the QAU of 3M Environmental Technology and Safety Services.

Test Material

Identification
Perfluorooctane sulfonic acid potassium salt (PFOS; T-6295)

Lot Number
The lot number will be maintained in the raw data.

Purity
Will be provided by the Sponsor

Stability
Responsibility of the Sponsor

Storage Conditions
Room temperature

Characteristics
Information on synthesis methods, composition, or other characteristics that define the test material is on file with the Sponsor.

Reserve (Archive) Samples
A reserve sample (approximately 5 g) of each lot will be taken and stored at room temperature. These samples will be transferred to the Sponsor after completion of the in-life phase to be retained in accordance with 40 CFR 792.195.

Disposition of Test Material
After authorization from the Sponsor, any remaining test material will be returned to:

Andrew M. Seacat, PhD
3M
Toxicology Services
Building 220-2E-02, 3M Center
St. Paul, Minnesota 55144-1000
Phone No.: 612.575.3161
Facsimile No.: 612.733.1733

Animals

Species
Rat

Strain
Crl:CD®(SD) IGS BR

Source
Charles River Laboratories, Inc., Raleigh, North Carolina

Age at Initiation of Treatment
Preferably 6 weeks of age, but not more than 8 weeks of age

Weight at Initiation of Treatment
100 to 300 g

Number and Sex
360 males and 360 females

Identification
Implantable microchip identification device

Husbandry

Housing
Individual (may be group-housed during acclimation)

Diet
Certified Rodent Diet #5002, meal (PMI Nutrition International) *ad libitum.* The diet is routinely analyzed by the manufacturer for nutritional components and environmental contaminants. Specified nutrient and contaminant analyses are on file at Covance-Madison.

Water
Ad libitum. Samples of the water are routinely analyzed for specified microorganisms and environmental contaminants. The results are on file at Covance-Madison.

Contaminants
There are no known contaminants in the diet or water at levels that might interfere with this study.

Environment
Environmental controls for the animal room will be set to maintain 18 to 26°C, a relative humidity of 30 to 70%, and a 12-hour light/12-hour dark cycle. The light/dark cycle may be interrupted to accommodate study-related activities.

Acclimation
At least 1 week

Randomization
Selection of animals for the study will be based on clinical observations and other data as appropriate. Animals will be assigned to treatment groups using a computerized blocking procedure designed to achieve body weight balance with respect to treatment groups. At the time of randomization, the weight variation of the animals of each sex used will not exceed ±2 standard deviations of the mean weight, and the mean body weight for each group of each sex will not be statistically different at the 5.0% probability level.

Justification
Rats historically have been used in safety evaluation studies and are recommended by appropriate regulatory agencies.

Group Designations and Dietary Levels

Group	Number of Animals Male	Female	Dietary Levels (ppm T-6295)
1 (Control)[a, b, c]	70	70	0
2 (Low)[b]	60	60	0.5
3 (Mid)[b]	60	60	2.0
4 (Mid-High)[b]	60	60	5.0
5 (High)[b, c]	70	70	20.0
6 (High Recovery)[d]	40	40	20.0

a The control animals will receive the basal diet only.
b Five animals/sex in Groups 1 through 5 will be sacrificed at Weeks 4 and 14 for hepatocellular proliferation rate measurements, biochemical analyses (palmitoyl-CoA oxidation), and histopathology (Week 14 only).
c Ten animals/sex in Groups 1 and 5 will be designated as interim sacrifice animals. These animals will be sacrificed after at least 52 weeks of treatment.
d Animals in Group 6 will be treated for at least 52 weeks, then treatment will be discontinued, and the animals will be observed for reversibility, persistence, or delayed occurrence of toxic effects for at least 52 weeks posttreatment. During recovery, the animals will receive basal diet only.

Dosing Procedures

Method of Administration

Dietary. Animals in Groups 1 through 5 will receive test diet for at least 104 weeks. Animals in Group 6 will receive test diet for 52 weeks only.

Reason for Dosing Route

The potential human exposure is by the oral route.

Dose Preparation

Before initiation of treatment, dose preparations of 0.5 ppm, 1 ppm, 2 ppm, and 20 ppm will be mixed.

At least every 4 weeks during the in-life phase, all dose preparations will be mixed according to the study-specific mixing procedure developed by Covance. Dose concentrations will be based on the T-6295 content of the test material as supplied.

All dose preparations will be stored at room temperature.

Retention Samples

Samples (approximately 100 g) will be taken from each dose preparation during the in-life phase and stored at room temperature. Unless used for analyses, these samples will be discarded at least 1 month after completion of the in-life phase.

Dose Analyses

By Covance using a method supplied by the Sponsor and validated by Covance

Homogeneity

Samples (approximately 100 g each) will be taken from the preparations mixed pretest and from preparations mixed for the first in-life interval. One sample each from the top, middle, and bottom of the dose preparations will be collected, divided into three subsamples for extraction and analysis, and analyzed for test material content. All samples will be stored at room temperature until analyzed within 7 days of mixing. Homogeneity analysis will be repeated if batch size changes by more than 30%.

Stability

One set of samples (approximately 100 g each) will be taken from the preparations mixed pretest, stored at room temperature for at least 28 days, then analyzed. Homogeneity samples collected from the middle of the pretest dose preparations will be analyzed within 2 days of mixing and used as the baseline value.

An additional set of samples (approximately 100 g each) will be taken from the preparations mixed for the first in-life interval, stored at room temperature for at least 32 days, then analyzed. Homogeneity samples collected from the middle of the dose preparations for the first in-life interval will be analyzed on the day of mixing and used as the baseline value.

Dose Confirmation

Samples (approximately 100 g each) will be collected from all dose preparations and analyzed in duplicate. Homogeneity samples collected from the middle of the dose preparations for the first in-life interval will be used for dose confirmation results. All samples will be stored at room temperature until analyzed.

Observation of Animals

Clinical Observations

Each animal will be observed twice daily (a.m. and p.m.) for mortality and moribundity; findings will be recorded as they are observed.

Once prior to treatment and weekly thereafter, each animal will be removed from its cage and examined; abnormal findings or an indication of normal will be recorded. The following information on each grossly visible or palpable mass will be recorded.

time of onset
location
size (small or large)
appearance
progression

Body Weights

Prior to treatment (at randomization), weekly for Weeks 1 through 17, once every 4 weeks thereafter, and at Week 105

Food Consumption

Weekly for Weeks 1 through 16 and once every 4 weeks thereafter

Clinical Pathology

Frequency and Number of Animals

Unscheduled Collection

When possible, a blood film will be made and held for possible future examination from animals sacrificed at an unscheduled interval.

Scheduled Collections

Hematology, serum chemistry, urinalysis, and urine chemistry will be done for 10 animals/sex/group In Groups 1 through 5 at Weeks 4, 14, 27, and 53.

A blood film will be made from animals at scheduled sacrifices after 52 and 104 weeks of treatment and held for possible future examination.

Method of Collection

Hematology, Serum Chemistry, Urinalyses, and Urine Chemistry

Animals will be fasted overnight; blood will be collected from a jugular vein. The anticoagulant will be potassium EDTA for hematology tests. Urine will be collected chilled overnight (approximately 16 hours).

Blood Films

Blood films will be taken as part of the necropsy procedure.

Tests

Hematology

red blood cell (erythrocyte) count	platelet count
hemoglobin	white blood cell (leukocyte) count
hematocrit	differential blood cell count
mean corpuscular volume	blood cell morphology
mean corpuscular hemoglobin	reticulocyte smear (made, but not examined)
mean corpuscular hemoglobin concentration	

Clinical Chemistry

glucose	alanine aminotransferase
urea nitrogen	gamma glutamyltransferase
creatinine	aspartate aminotransferase
total protein	calcium
albumin	inorganic phosphorus
globulin	sodium
cholesterol	potassium
total bilirubin	chloride

Urinalysis

appearance	glucose
volume	ketones
specific gravity	bilirubin
pH	blood
protein	microscopic examination of sediment
urobilinogen	

Urine Chemistry

sodium	16 hour excretion of:
potassium	sodium
	potassium

Serum PFOS Analyses

Frequency and Number of Animals

Five animals/sex in Groups 1 through 5 during Weeks 4 and 14 (animals selected for hepatocellular proliferation and biochemical analyses), five animals/sex/group from Groups 1 and 5 after at least 52 weeks of treatment (from animals selected for interim sacrifice), and five animals/sex/group from Groups 1 through 5 at terminal sacrifice

Method of Collection

Animals will be fasted overnight; blood (approximately 2 mL) will be collected from a jugular vein. Samples will be collected without anticoagulant.

Sample Handling

Blood samples will be allowed to clot at room temperature and centrifuged. Serum samples will be harvested and stored in a freezer set to maintain -60 to -80°C. Samples will be packed on dry ice and shipped to:

Kris J. Hansen, PhD
3M Environmental Technology and Safety Services
935 Bush Avenue
Building 2-3E-09
St. Paul, Minnesota 55133-3331
Telephone No.: 612.778.6018
Facsimile No.: 612.778.6176

Serum samples will be analyzed for PFOS and metabolites by the Sponsor. Results will be provided for inclusion in the final report.

Termination

Unscheduled Sacrifices and Deaths

Necropsies will be done. Animals to be sacrificed will be anesthetized with sodium pentobarbital, weighed, and exsanguinated. A blood film will be taken as part of the necropsy procedure for sacrificed animals.

Scheduled Sacrifices

Interim Sacrifices

During Week 4, five animals/sex/group from Groups 1 through 5 will be fasted overnight, bled for serum samples, anesthetized with sodium pentobarbital, weighed, and exsanguinated. The abdominal cavity of each animal will be opened, the liver will be removed and weighed, and liver samples will be collected. Animals will be discarded after liver collection.

During Week 14, five animals/sex/group from Groups 1 through 5 will be fasted overnight, bled for serum samples, anesthetized with sodium pentobarbital, weighed, exsanguinated, and necropsied.

After at least 52 weeks of treatment, 10 animals/sex/group from Groups 1 and 5 will be fasted overnight, bled for serum samples (five animals/sex/group) anesthetized with sodium pentobarbital, weighed, exsanguinated, and necropsied. The liver will be weighed and frozen liver samples will be collected. A blood film will be taken as part of the necropsy procedure.

Terminal Sacrifice

After at least 104 weeks of treatment, the remaining animals will be fasted overnight, bled for serum samples (five animals/sex/group), anesthetized with sodium pentobarbital, weighed, exsanguinated, and necropsied. A blood film will be taken as part of the necropsy procedure.

Recovery Sacrifice

After at least 52 weeks of treatment and 52 weeks without treatment, the remaining animals in Group 6 will be fasted overnight, then anesthetized with sodium pentobarbital, weighed, exsanguinated, and necropsied.

Postmortem Procedures

Necropsy

The necropsy will include an examination of the external features of the carcass; all external body orifices; the abdominal, thoracic, and cranial cavities; organs; and tissues.

Cell Proliferation Tissue Collection and Immunohistochemical Evaluation

At the Week 4 and 14 interim sacrifices, representative samples of the left lateral lobe of the liver and any macroscopic lesions of the liver will be collected and preserved in zinc formalin.

After fixation, each sample of liver will be embedded in paraffin, and the paraffin blocks will be shipped to:

Sandra R. Eldridge, PhD
Pathology Associates International
15 Worman's Mill Court, Suite I
Frederick, Maryland 21701
Telephone No.: 301.663.1644, ext. 2201
Facsimile No: 301.663.8994

Proliferation cell nuclear antigen (PCNA) evaluation will be done on the samples. In addition, liver sections will be stained with hematoxylin and eosin and examined microscopically. Results will be provided for inclusion in the final report.

Palmitoyl-CoA Oxidase Tissue Collection and Analyses

At the Week 4 and 14 interim sacrifices, a sample (approximately 500 mg) of the right lateral lobe of the liver will also be collected from each animal and flash-frozen in liquid nitrogen. The liver tissue will be stored in a freezer set to maintain -60 to -80°C until analyzed by Covance for palmitoyl-CoA oxidase activity.

Organ Weights

At the scheduled sacrifices, the following organs (when present) will be weighed; paired organs will be weighed separately:

adrenal (2)	ovary (2)
brain	spleen
kidney (2)	testes
liver	thyroid (2) with parathyroid
lung	uterus with cervix

Organ-to-body weight percentages and organ-to-brain weight ratios will be calculated.

Bone Marrow Smear

From the femur of each animal at scheduled sacrifices only; made but not examined

Liver PFOS Analysis

A portion of the liver will be collected from five animals/sex/group from Groups 1 through 5 at the Week 4 and 14 interim sacrifices, from five animals/sex/group from Groups 1 and 5 at the Week 79 interim sacrifice, and from five animals/sex/groups from Groups 1 through 5 at the terminal sacrifice and flash-frozen in liquid nitrogen and stored in a freezer set to maintain -60 to -80°C. Samples will be packed on dry ice and shipped to Kris J. Hansen, PhD, 3M Environmental Technology and Safety Services. Liver samples will be analyzed for PFOS and metabolites by the Sponsor. Results will be provided for inclusion in the final report.

Tissue Preservation

The following tissues (when present) from each animal will be preserved in 10% neutral-buffered formalin:

adrenal (2)	eye (2)
brain	femur with bone marrow (articular
cecum	surface of the distal end)
cervix	Harderian gland
colon	heart
duodenum	ileum
epididymis (2)	jejunum
esophagus	kidney (2)

lesions
liver
lung with mainstem bronchi
lymph node (mesenteric)
mammary gland (females only)
ovary (2)
pancreas
pituitary
prostate
rectum
salivary gland [mandibular (2)]
sciatic nerve
seminal vesicle (2)
skeletal muscle (thigh)
skin
spinal cord (cervical, thoracic, and lumbar)
spleen
sternum with bone marrow
stomach
testis (2)
thymus
thyroid (2) with parathyroid
trachea
urinary bladder
uterus
vagina

Histopathology

Tissues (as appropriate) from each animal in Groups 1, 5, and 6 sacrificed at the Week 53 interim sacrifice and terminal sacrifice and from each animal that dies or is sacrificed at an unscheduled interval will be embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically. Adrenals, brain, eyes, kidneys, liver, mesenteric lymph node, pancreas, spleen, testes, and ovaries from the animals necropsied at the Week 14 interim sacrifice will also be embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically.

Macroscopic lesions will also be examined microscopically from each animal in Groups 2, 3, and 4 sacrificed at terminal necropsy.

Reports

One copy of the draft report will be sent to the Sponsor. The report will include the following information:

Experimental Design and Methods

Results

dose analyses
mortality
clinical observations
body weights

body weight changes
food consumption
test material consumption
clinical pathology results
palmitoyl CoA oxidase activities
serum PFOS and metabolite values (provided by the Sponsor)
liver PFOS and metabolite values (provided by the Sponsor)
macroscopic observations
microscopic observations
cell proliferation assessments (provided by the Sponsor's designee)

Statistical Evaluation

Levene's test will be done to test for variance homogeneity. In the case of heterogeneity of variance at $p \leq 0.05$, transformations will be used to stabilize the variance. Comparison tests will take variance heterogeneity into consideration.

One-way analysis of variance (ANOVA) will be used (if applicable) to analyze body weights, body weight changes, food consumption, continuous clinical pathology values, palmitoyl CoA oxidase activities, and organ weight data. If the ANOVA is significant, Dunnett's t-test will be used for control versus treated group comparisons.

If the ANOVA shows significance for body weights at Week 1, one-way analysis of covariance (ANCOVA) will be used to analyze body weights, with initial body weights as the covariate. If the ANCOVA is significant, covariate-adjusted means will be used for control versus treated group comparisons.

Group comparisons (Groups 2 through 6 versus Group 1) will be evaluated at the 5.0% two-tailed probability level. Only data collected on or after the first day of treatment will be analyzed statistically.

Adjusted survival data are analyzed by the National Cancer Institute (NCI) lifetable package. The tests include: Graphical (Kaplan-Meier product-limit estimation curves), Cox-Tarone binary regression methods for trend and heterogeneity, and Gehan-Breslow nonparametric methods for trend and heterogeneity.

Non-neoplastic lesions are analyzed by the Cochran-Armitage test for trend and the Fisher-Irwin exact test for heterogeneity.

Incidental tumors are analyzed by Dinse-Lagakos logistic prevalence methods for trend and heterogeneity. Rapidly lethal and palpable tumors are analyzed in the same manner as survival.

In the cases where the study pathologist can assign particular occult neoplastic lesions as the cause of death in the animals, such information will be taken into appropriate analysis.

At the end of 1 year after issuance of the audited draft report, if no requested revisions or instructions to finalize have been communicated by the Sponsor, then the audited draft report will be considered 'final' and issued as the final report, signed by the study director, and submitted to the Sponsor.

Any modifications or changes to the audited draft report requested 1 year after issuance will be performed at additional cost to the Sponsor.

Two copies of the signed final report (one unbound and one bound) will be sent to the Sponsor.

Record Retention

All raw data, documentation, records, protocol, specimens, and final report generated as a result of this study will be archived in the storage facilities of Covance-Madison for a period of 1 year following submission of the final report to the Sponsor. One year after submission of the final report, all of the aforementioned materials will be sent to the Sponsor, and a return fee will be charged. The Sponsor may elect to have the materials retained in the Covance archives for an additional period of time, and Covance will charge a storage fee. If the Sponsor chooses to have Covance dispose of the materials, a disposal fee will be charged. All raw data stored on magnetic media will be retained by Covance.

PCNA evaluation data and paraffin blocks and tissue slides for PCNA will be retained by Pathology Associates International.

Liver and serum samples sent to the Sponsor will be retained by the Sponsor.

PROTOCOL APPROVAL

Andrew M. Seacat

Andrew M. Seacat, PhD
Study Monitor
3M

4/21/98
Date

Peter J. Thornford

Peter J. Thornford, PhD
Study Director
Covance Laboratories Inc.

4/15/98
Date