

DOW CORNING

RECEIVED
EPA/OPIC

2007 OCT -4 AM 11:22

October 2, 2007

Document Control Office (7407)
TSCA Data Processing Center - CBIC
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
Room 6428
1200 Pennsylvania Avenue, NW
Washington, DC 20460

Contains No CBI

CONTAIN NO CP'



Attn: TSCA Docket Clerk

Re: For Your Information Submission:

The enclosed information is submitted on behalf of Dow Corning Corporation, Midland, Michigan, 48686-0994, on a For-Your-Information (FYI) basis as a follow-up to submissions made concerning hexamethyldisiloxane (HMDS), which chemical substance was the subject of a health and safety data rule issued under Section 8(d) of the Toxic Substances Control Act (TSCA) and with an effective date of June 14, 1993 (sunset date June 30, 1998), as codified at 40 CFR 716 (Health and Safety Data Reporting). The information presented in this submission was generated as part of our Siloxane Research Program. This program was the subject of a memorandum of understanding, dated April 9, 1996, between Dow Corning and EPA.

Listed Chemical Substance:

107-46-0 Hexamethyldisiloxane (HMDS)



Final Study Report:

Non-Regulated Study: Hexamethyldisiloxane (HMDS): Determination of the Reverse Binding of HMDS/Metabolites to Alpha 2u-Globulin in Male Fischer 344 Rats Following Oral Gavage Administration

Dow Corning Corporation
2007-I0000-57893
September 21, 2007

MA #
307300

RECEIVED
EPA T-0110

2007 OCT -4 AM 11: 22

Manufacturer:

Dow Corning Corporation
PO Box 994
2200 West Salzburg Road
Midland, Michigan 48686-0994

For purposes of this TSCA For-Your-Information (FYI) submission, the general INTERNAL designation on the attached health and safety report is waived by Dow Corning.

If you require further information regarding this submission, please contact Michael Thelen, Manager of U.S. EPA Regulatory Affairs, at 989-496-4168 or at the address provided herein.

Sincerely,



Kathleen P. Plotzke
Director, Health and Environmental Sciences
(989) 496-8046

RECEIVED
3821 0412

2007 OCT -4 1:51:22

DOW CORNING CORPORATION
HEALTH & ENVIRONMENTAL SCIENCES
TECHNICAL REPORT

Report Number: 2007-I0000-57893

Title: NON-REGULATED STUDY:
Hexamethyldisiloxane (HMDS): Determination of
the Reversible Binding of HMDS/Metabolites to
Alpha 2u-Globulin in Male Fischer 344 Rats
Following Oral Gavage Administration.

Study Number: 10461-101

Test Article: Hexamethyldisiloxane (HMDS)

Study Leader: Jeanne Y. Domoradzki, M.S., Ph.D., D.A.B.T.

Sponsor: Dow Corning Corporation
2200 W. Salzburg Road
Auburn, MI 48611

HES Management: Paul A. Jean, Ph.D.
Team Leader, Toxicology

Testing Facility: Dow Corning Corporation
Health and Environmental Sciences
Auburn, MI 48611

Study Completion Date: September 21, 2007

Security Statement: DOW CORNING INTERNAL INFORMATION:
This report may be reproduced and shared with any Dow
Corning employee. The director of the issuing department
must approve distribution outside the corporation. When this
INTERNAL report is no longer needed, it must be destroyed
in a manner that safeguards against unauthorized disclosure.

2007 © Dow Corning Corporation

All rights reserved. No part of this work may be reproduced or transmitted in any form by any means, electronic or mechanical, including but not limited to photocopying or recording, or by any information storage or retrieval system without permission in writing from Dow Corning Corporation.

MJ#
307300

TABLE OF CONTENTS		Page
TITLE PAGE		1
TABLE OF CONTENTS		2
LIST OF TABLES AND FIGURES		3
LIST OF APPENDICES		4
1 ABSTRACT.....		5
2 APPROVAL SIGNATURES.....		7
3 STUDY INFORMATION		8
4 STUDY PURPOSE.....		9
5 TEST ARTICLE / SUBSTANCE / ITEM.....		9
6 CARRIER		11
7 TEST SYSTEM		11
8 JUSTIFICATION FOR SELECTION OF TEST SYSTEM.....		12
9 METHOD OF RANDOMIZATION.....		12
10 HOUSING AND MAINTENANCE.....		12
11 ANIMAL WELFARE ACT COMPLIANCE		13
12 ROUTE AND RATIONALE OF TEST ARTICLE ADMINISTRATION		13
13 ORGANIZATION OF TEST GROUPS AND EXPOSURE LEVELS		15
14 TREATMENT REGIMEN AND KEY EVENTS/ACTIVITIES		16
15 METHOD OF EUTHANASIA/TERMINAL PROCEDURE		17
16 TEST SYSTEM OBSERVATION		17
17 SAMPLE PROCESSING AND ANALYSIS		18
18 SAMPLE IDENTIFICATION AND STORAGE		19
19 DATA ANALYSIS.....		19
20 RESULTS		20
21 DISCUSSION		23
22 CONCLUSIONS.....		27
23 ARCHIVE.....		27
23 REFERENCES		28

LIST OF TABLES	Page
Table 1 – Summary of Clinical Observations.....	30
Table 2 - Summary of Weekly Mean Body Weights (grams).....	40
Table 3 - Summary of Weekly Mean Body Weight Gains (grams).....	41
Table 4 - Concentration of Parent and Radioactivity and Percentage of Parent/Radioactivity at 2 h Post-Dosing.....	42
Table 5 - Percent Peak Area Summary of the Alpha 2u-globulin Peak Region from HIC HPLC/DAD Analysis.....	43
Table 6 - Percent Peak Area Summary of ¹⁴ C-HMDS and/or Metabolites from HIC HPLC/RAD Analysis.....	44

LIST OF FIGURES	Page
Figure 1 – Representative DAD Chromatogram of Male Kidney Cytosol Profile	45
Figure 2 - Representative DAD Chromatogram of Female Kidney Cytosol Profile.....	46
Figure 3 - Dot Blot of Representative Male versus Female Kidney Cytosol Fractions Collected From HIC.....	47
Figure 4 – Representative RAD Chromatogram of Male Kidney Cytosol Profile	48
Figure 5 - Representative RAD Chromatogram of Female Kidney Cytosol Profile.....	49
Figure 6 - Representative DAD Chromatogram of Male Kidney Cytosol Profile after Incubation with DMSO (Negative Control).....	50
Figure 7 – Representative DAD Chromatogram of Male Kidney Cytosol Profile after Incubation with D-Limonene Oxide	51
Figure 8 - Representative RAD Chromatogram of Male Kidney Cytosol Profile after Incubation with DMSO (Negative Control).....	52
Figure 9 - Representative RAD Chromatogram of Male Kidney Cytosol Profile After Incubation with D-Limonene Oxide	53

LIST OF APPENDICES

Page

Appendix A – Individual Clinical Observations and Detailed Clinical Observations.....	A1-A16
Appendix B – Individual Body Weights (grams).....	B1-B6
Appendix C – Individual Body Weight Gain (grams)	C1-C6
Appendix D - Contributing Scientist Report: Pathology	D1-D21
Appendix E – Contributing Scientist Report: Analytical – Group 5	E1-E21
Appendix F - Contributing Scientist Report: Reversible Binding – Group 5.....	F1-F21

1 ABSTRACT

Male Fischer 344 rats were administered HMDS (hexamethyldisiloxane) by oral gavage in a corn oil vehicle at 0, 10, 100 and 1000 mg/kg body weight for 28 days; groups 1-4. In addition, one group of animals consisting of male and female rats was administered 1000 mg HMDS/kg bw for 27 days and ¹⁴C-HMDS was administered at the same dose level on day 28; group 5. There was a reduction in body weight gain in group 4 and 5 male animals as compared to control animals indicating the dose level of 1000 mg/kg for 28 days caused some systemic toxicity. Kidneys from animals in groups 1-4 were examined microscopically for the presence of hyaline droplet formation that is indicative of alpha 2u-globulin nephropathy. In the group administered HMDS at 10 mg/kg/day for 28 days, the amount and histomorphology of protein droplet accumulation in the cortex did not appear different from controls. A very subtle change was observed in the group receiving 100 mg/kg/day and the nephropathy in this group was graded as minimal. Alpha 2u-nephropathy was graded as moderate in all sections examined of group 4, 1000 mg/kg/day, animals.

Kidney cytosol was prepared from the male and female F344 rats administered HMDS by oral gavage upon improvement of a published procedure to minimize loss of volatile/lipophilic siloxanes. A new approach using Hydrophobic Interaction Chromatography (HIC) was developed to isolate the alpha 2u-globulin protein peak region from other proteins in male and female rat kidney cytosol samples. A dot blot using commercially available polyclonal antibodies raised against purified rat alpha 2u-urinary globulin was used to positively identify the presence of alpha 2u-globulin protein in the fraction isolated by HIC. Alpha 2u-globulin was present in the male rat kidney eluent HIC fraction and not in the female rat kidney fraction. Radioactivity co-eluting with the alpha 2u-globulin peak region was then monitored to determine the binding of HMDS or metabolite to the protein. Further experimentation demonstrated that D-limonene oxide, a chemical with a high affinity for alpha 2u-globulin, displaced or reversed the binding of HMDS and/or metabolites to alpha 2u-globulin in samples of male rat kidney cytosol from HMDS treated rats.

Demonstration of reversible binding of HMDS or metabolites to alpha 2u-globulin in male rat kidney samples from HMDS treated rats, along with the present and previous observations of increased hyaline droplet formation, male rat specific kidney tumors, and the presence of alpha 2u-globulin in kidney sections detected by immunohistochemistry

shows that this species specific mechanism, alpha 2u-nephropathy, of toxicity is responsible for the rat kidney neoplasia observed in a previous chronic/carcinogenicity study with HMDS. Therefore, this mechanism of kidney tumor formation in male rats following administration of HMDS is species specific and not relevant to human cancer risk assessment.

2 APPROVAL SIGNATURES

The undersigned have read and approved this report:



Paul A. Jean, Ph.D.
Team Leader, Toxicology
Health and Environmental Sciences

21 Sept 07
Date



Jeanne Y. Domoradzki, Ph.D., D.A.B.T.
Study Leader
Health and Environmental Sciences

September 21, 2007
Date

3 STUDY INFORMATION

Study Initiation Date: 04/10/07

Experimental Start Date: 04/12/07

Experimental Termination Date: 07/31/07

Study Completion Date: September 21, 2007

Study Number: 10461-101

Study Title: NON-REGULATED STUDY:
Hexamethyldisiloxane (HMDS):
Determination of the Reversible Binding of
HMDS/Metabolites to Alpha 2u-Globulin in
Male Fischer 344 Rats Following Oral
Gavage Administration.

Testing Facility: Health and Environmental Sciences (HES)
Dow Corning Corporation
2200 West Salzburg Road
Midland, MI 48686-0994

Study Leader: Jeanne Y. Domoradzki, Ph.D., D.A.B.T

Team Leader, Toxicology: Paul A. Jean, Ph.D.

Study Coordinator: Chris Sushynski, B.S.

Contributing Scientists: Debra A. McNett, B.S.
Jacob Sushynski, B.S.
Lisa Thackery, B.S.
James W. Crissman, D.V.M., Ph.D.,
D.A.C.V.P.

Manager, Analytical Chemistry: Roy A. Campbell, B.S.

4 STUDY PURPOSE

The objective of this study was to evaluate alpha 2u-nephropathy induction and the reversible binding of alpha 2u-globulin to HMDS and/or metabolites in male rat kidneys following oral gavage administration of HMDS in a corn oil vehicle to male and female (control) animals for 28 days.

5 TEST ARTICLE / SUBSTANCE / ITEM

The Study Leader reviewed results of test article characterization and that review is documented in the study file.

Identification:	Hexamethyldisiloxane (HMDS) (Supplied as Dow Corning® 200 Fluid, 0.65cst) ZMAT Number: 1013084
Lot No.:	0001524145
CAS No.:	107-46-0
Physical Description:	Colorless liquid
Source:	Dow Corning Corporation 2200 W. Salzburg Road Auburn, MI 48611
Chemical stability:	Chemically stable
Storage Conditions:	Room temperature
Expiration Date:	2-13-2008
Purity:	99.92 ± 0.001%
Solubility:	Acetone and heptane
Chemical characterization:	Dow Corning Study Number 9862-101
Archive:	A reserved sample was not retained.

Identification: ^{14}C - Hexamethyldisiloxane (^{14}C -HMDS)

Reference No.: 20785-7

CAS No.: Not Available

Physical Description: Colorless liquid

Source: Dow Corning Corporation
2200 W. Salzburg Road
Auburn, MI 48611

Chemical stability: Material degrades if in contact with strong acids and bases. In, general radioactive materials undergo degradation by radiolysis in storage.

Storage Conditions: $-80^{\circ}\text{C} \pm 10^{\circ}\text{C}$ (until final dilution with unlabeled HMDS)

Expiration Date: 10-10-2008

Specific Activity: 73.879 ± 1.43 mCi/g

Radiochemical Purity: $99.52 \pm 0.095\%$

Solubility: Common organic solvents

Chemical characterization: Dow Corning Study Number 10255

Archive: Reserved sample will not be retained

^{14}C -HMDS was diluted with unlabeled test article and a new reference number was assigned by HES test article control and characterization group or designee. Specific activity of the resultant ^{14}C - test article was determined to be 0.06554 mCi/g HMDS.

6 CARRIER

Identification: Corn Oil
Batch Number: 074K0025
Expiration Date: April 6, 2010
Source: Sigma-Aldrich
3050 Spruce Street
Saint Louis, MO 63103
Physical Description: Deep yellow clear liquid, refer to MSDS
Stability: Stable, refer to MSDS
Purity: As provided by the manufacturer
Storage Conditions: Store in sealed container

7 TEST SYSTEM

Species: *Rattus norvegicus*
Strain: Fischer 344
Source: Charles River Laboratories, Inc., (address to be documented in the study records)
Age: 10 weeks minimum at experimental start
Body weight: Females: 150-175 g minimum at experimental start
Males: 200-250 g minimum at experimental start
Sex and number used on study: Females: 10
Males: 30
Number of groups: 5
Identification method: Transponders

8 JUSTIFICATION FOR SELECTION OF TEST SYSTEM

The species and strain of animal is recognized as appropriate for toxicity studies and Fischer 344 female and male rats have previously been used in pharmacokinetic and metabolism studies of various silicone materials. Previous studies with HMDS have indicated alpha 2u-globulin nephropathy in male Fischer 344 rats. Female rats were used in this study as controls since female rats do not develop alpha 2u-globuline nephropathy. The number of animals used provided adequate kidney samples for analysis of reversible binding of alpha 2u-globulin with HMDS and/or its metabolites in the kidney and confirmation of alpha 2u-globulin nephropathy dose response in male rats.

9 METHOD OF RANDOMIZATION

Extra animals were ordered and acclimated prior to randomization. After release from quarantine/acclimation, rats were assigned to test groups based on a weight-stratified randomization process. At randomization the sex specific weight variation of animals in each group did not exceed $\pm 20\%$ of the mean weight for that group. Disposition of all animals not utilized in the study are maintained on file at the Testing Facility.

10 HOUSING AND MAINTENANCE

10.1 Animal Receipt and Quarantine/Acclimation

Animal Resource personnel inspected each animal upon receipt and at least once daily during the quarantine period. Animals judged to be in good health and suitable as test animals were quarantined/acclimated for five days. The attending veterinarian, or designee, examined all animals before release from quarantine/acclimation and documented the general state of animal health.

10.2 Animal Housing

Animals were individually housed in suspended wire-mesh cages elevated over Bed-O'Cobs® litter, during quarantine and throughout the course of the study. The Study Leader reviewed the analysis of the litter.

10.3 Enrichment

Gnaw Pucks® were provided as an enrichment device for the animals.

10.4 Environmental Conditions

Animals were housed in an environmentally controlled animal room (12-hour fluorescent-light/dark cycle, 17.8-26.1°C, 30-70% humidity, 10-20 air changes per hour). Temperature and humidity were monitored continuously, recorded every 15 minutes using a HOBO® Data Logger (Onset Computer, Bourne, MA), and manually recorded twice a day on weekdays and at least once per day on weekends and holidays. The Study Leader reviewed the most recent verification of air changes.

10.5 Basal Diet

Certified Rodent Diet #5002, PMI® Nutritional International Inc., St. Louis, MO, were offered ad libitum. Manufacturer's periodic analyses of the certified feed for the presence of heavy metals and pesticides maintained on file at the Testing Facility was reviewed by the Study Leader to ensure that none are present in concentrations that would be expected to affect the outcome of the study.

10.6 Drinking Water

Municipal water, further purified by reverse osmosis (RO) was available ad libitum. Drinking water is monitored routinely and also analyzed on a semi-annual basis. The Study Leader reviewed the most recent analysis results maintained on file at the Testing Facility to ensure that no contaminants are present in a concentration expected to interfere with the integrity of the study.

11 ANIMAL WELFARE ACT COMPLIANCE

This study complies with all applicable sections of the final rules of the Animal Welfare Act regulations (9 CFR, Part 1, 2, and 3) and was approved by the Institutional Laboratory Animal Care and Use Committee (LACUC) before animals were ordered.

12 ROUTE AND RATIONALE OF TEST ARTICLE ADMINISTRATION

12.1 Route

Test article was administered via oral gavage for 28 consecutive days.

12.2 Rationale

This route is an accepted method of administration of test article in pharmacokinetics and metabolism studies. The highest dose level and the duration of administration were determined based on the results of a previously conducted study (Dow Corning report number 1990-I0000-35105). At this dose and duration of administration, hyaline droplet nephropathy which is consistent with alpha 2u-nephropathy was expected to be observed.

12.3 Dosing Solution Preparation and Analysis

Reversible binding of HMDS to alpha 2u-globulin: Unlabeled test article was used as supplied and prepared in a corn oil solution for oral gavage administration days 1 through 27. ¹⁴C-labeled HMDS test article in a corn oil solution was administered on day 28 to the high dose group (group 5), 1000 mg HMDS/kg bw, at a targeted radioactivity level of 200 uCi per animal.

Alpha 2u-nephropathy: Unlabeled test article was used as supplied and prepared in a corn oil solution for oral gavage administration days 1 through 28.

Preparation

The non-radiolabeled dosing solution was prepared once and stored at room temperature. Previous data indicate (Study Number 10210-101) that HMDS is stable in a corn oil solution for at least five months. The radiolabeled dose solution was prepared the day prior to administration by adding carrier to volume to a weighed amount of HMDS and ¹⁴C-HMDS (~73.879 mCi/g). Calculations and preparation methods for dosing solutions were documented in the raw data.

Analysis

Dosing solution analyses were not performed to verify concentration, stability, and homogeneity of the test substance in carrier. A verification of the radiochemical purity for the labeled dosing solution was evaluated using HPLC with radiomatic detection. Specific activity of the labeled dosing solution was determined by liquid scintillation analysis on the day of preparation as well as prior to initiation of oral gavage administration.

12.4 Storage conditions

The dosing solution preparations were stored at room temperature.

12.5 Administration of doses

Dosing solutions were administered by oral gavage with a 3-4 inch, 15-18 gauge animal feeding needle and syringe at a volume of 2 mL/kg of body weight. Volume administered was based upon the most recent individual animal weight.

13 ORGANIZATION OF TEST GROUPS AND EXPOSURE LEVELS

Group Number	Group Description	Number of Animals	Treatment	mg HMDS/kg bw	Collections post final HMDS administration (Hours)	Treatment (Days)
1	Histopathology Controls	4 M	Corn oil	0	Kidneys (2 hours)	28
2	Histopathology	5 M	HMDS	10	Kidneys (2 hours)	28
3	Histopathology	5 M	HMDS	100	Kidneys (2 hours)	28
4	Histopathology	4 M	HMDS	1000	Kidneys (2 hours)	28
5	Reversible Binding	10 F & 10 M	HMDS	1000	Kidneys and blood (2 hours)	28

One kidney from each animal in Group 5 was processed to determine radioactivity and parent. Blood was also collected from each animal for radioactivity and parent determination.

13.1 Histopathology Control (Group 1)

These animals were administered corn oil for a total of 28 consecutive days and served as background controls for the histopathology portion of the study. These animals were sacrificed at 2 hours post-final administration. Kidneys were collected and processed for histopathology.

13.2 Histopathology (Groups 2, 3, and 4)

These animals were administered HMDS in corn oil for 28 consecutive days at 10, 100 and 1000 mg/kg. On day 28 the animals were sacrificed 2 h after this final administration. Kidneys were collected and processed for histopathology.

13.3 Reversible Binding (Group 5)

These animals were administered HMDS in corn oil for 27 consecutive days at 1000 mg/kg. On day 28 the animals received ¹⁴C-HMDS in corn oil (target of 200 uCi/animal) and were sacrificed 2 h after this final administration. Terminal blood was collected. Kidneys were collected and frozen on dry ice and remained at -80°C until time of analysis.

Rationale for Selection of 2 h Sacrifice Time

The PBPK model for HMDS developed by Dobrev *et al.*, 2003 was used to approximate the time of sacrifice following an oral gavage administration of 1000 mg HMDS/kg bodyweight to rats. The parameters for metabolism, blood flow rates and tissue blood partition coefficients used in the model were the ones described in the paper. It was determined that an early sacrifice of 2 h post-administration of the oral dose would give blood concentrations of HMDS sufficient for analytical sensitivity needed to determine reversible binding. The model predicted HMDS concentration in blood was 25 ug/g blood.

14 TREATMENT REGIMEN AND KEY EVENTS/ACTIVITIES

14.1 Treatment Regimen

Group 1 received corn oil only for a total of 28 consecutive days.

Groups 2, 3 and 4 received 28 consecutive days of 10, 100 or 1000 mg HMDS/kg bw in corn oil.

Group 5 received 27 consecutive days of 1000 mg HMDS/kg bw in corn oil followed by oral gavage administration of ¹⁴C-HMDS in corn oil (target of 200 uCi/animal) on day 28.

14.2 Key Events/Activities

Study day -1:	Body weights and randomization.
Study day 1-27:	Body weights (once per week); oral gavage administration groups 1-5.
Study day 28:	Body weights, oral gavage administration groups 1-5. Animals sacrificed at 2 hours post-administration. Body weights taken. Kidneys collected from 10 animals/sex for alpha 2u-globulin reversible binding determinations. Kidneys collected from four-five animals/group for determination of alpha 2u-globulin nephropathy.

15 METHOD OF EUTHANASIA/TERMINAL PROCEDURE

15.1 Scheduled animal death

Animals from groups 1-4 were euthanized by CO₂ asphyxiation. Animals in group 5 were anesthetized with Isoflurane® and euthanized by exsanguination.

15.2 Unscheduled animal death

Measures were taken to minimize discomfort or pain for all animals. The decision to euthanize an animal was made by the Study Leader. Animal D-4099 was euthanized by CO₂ asphyxiation since labored respiration was observed. Tissues and blood were not collected from this animal.

16 TEST SYSTEM OBSERVATION

16.1 Mortality/Morbidity/Moribundity

All animals were observed at least once daily in their cages for mortality, morbidity, and moribundity by study personnel throughout the completion of the in-life phase of the study. No animals were found dead during the study.

16.2 Parameters measured

Individual body weights were measured and recorded for each animal on the day of randomization, prior to initiation of the animals' oral gavage administration, once per

week and on scheduled sacrifice days prior to euthanasia. Radioactivity content in blood and kidney were determined and as well as the concentration of parent HMDS. Measurements of reversible binding of HMDS and/or metabolites to alpha 2u-globulin were conducted. Kidneys were examined for alpha 2u-globulin nephropathy.

17 SAMPLE PROCESSING AND ANALYSIS

17.1 Alpha 2u-globulin nephropathy (Groups 1, 2, 3 and 4)

Tissues and organs required for histopathological examination were taken and preserved in fixative, 10% Neutral Buffered Formalin. Tissues were processed routinely, embedded in paraffin, sectioned at approximately 5 microns and stained with hematoxylin and eosin for histological examination.

17.2 Reversible Binding (Group 5)

The animals were anesthetized with Isoflurane® and exsanguinated by collecting a maximum volume of blood. As quickly as possible following collection of blood, the kidneys were removed, capsule removed, blotted of excess blood and placed into pre-weighed containers. The collected tissues were weighed and frozen on dry ice. The kidney samples were analyzed for the reversible binding of HMDS and/or metabolites to alpha 2u-globulin. Selected kidneys were homogenized in a Tris Buffer solution and analyzed by HPLC on a HIC (Hydrophobic Interaction Chromotography) column. Portions of the homogenate were incubated with D-limonene oxide and subsequently analyzed by HPLC on a HIC column to determine if competitive binding to the protein occurs. The methodology for these analyses is included in the study file and the attached Contributing Scientist Report (Appendix F).

Selected kidneys were placed in pre-weighed vials containing known amounts of internal standard and THF for extraction. An aliquot of each extract was analyzed by GC/MSD according to the method "Procedure for Determination of HMDS in Biological Matrices (Blood and Tissues)". Also, an aliquot of the extract was analyzed by liquid scintillation counting (LSC) for radioactivity content. Additionally the extracted pellet was solubilized using TEAH, the weight of the pellet and TEAH was obtained. Duplicate aliquots of the solubilized pellet were weighed and analyzed by LSC for radioactivity analysis. The methodology for these analyses is included in the study file and the attached Contributing Scientist Report (Appendix E).

18 SAMPLE IDENTIFICATION AND STORAGE

Samples collected were identified by study number, test system or specimen number, target time point if applicable, identity/name of sample, date of collection and storage conditions.

Samples for the reversible binding portion of the study were stored under the following conditions:

Blood $5 \pm 4^{\circ}\text{C}$,
Kidney (reversible binding) $-80 \pm 10^{\circ}\text{C}$.

Upon collection of the kidneys for microscopic examination, samples were stored at room temperature

19 DATA ANALYSIS

19.1 Parameters evaluated

Body Weight Measurements

Individual body weights were measured and recorded for each animal on the day of randomization, prior to initiation of oral gavage administration, once per week and on scheduled sacrifice day prior to euthanasia.

Macroscopic Examination

Macroscopic examination of the kidneys was conducted for groups 1-4.

Microscopic Examination

Light microscopic kidney morphology was evaluated for protein droplet nephropathy.

Tissue Concentration

For the reversible binding portion of the study kidney weights and blood weights were determined at the time of tissue collection. Parent HMDS and total radioactivity were measured in the blood and kidneys as described above. The concentration of radioactivity in whole blood and tissues was recorded as $\mu\text{g } ^{14}\text{C-HMDS}$ equivalents per gram sample. The calculation of $^{14}\text{C-HMDS}$ equivalents was based upon the specific activity of the

dosing solution. The concentration of HMDS in whole blood and tissues was reported as ug HMDS per gram sample.

Reversible binding of HMDS to alpha 2u-globulin

Kidney extracts were analyzed to confirm the presence of alpha 2u-globulin and to detect the radioactivity bound to alpha 2u-globulin. Kidney extracts were incubated with D-limonene oxide. D-limonene oxide because of its high affinity for alpha 2u-globulin should displace bound HMDS/metabolite from the protein and indicate a reversible binding process.

19.2 Statistical methods

Numerical data obtained during the conduct of the study were processed using Microsoft Excel™ and subjected to calculation of parameters indicated above, this included group mean values and standard error of the mean, where appropriate. Statistical analysis of the data was not conducted.

20 RESULTS

20.1 Mortality and Morbidity

All but one animal survived to its scheduled necropsy. Animal D-4099, group 5, was sacrificed due to labored respiration which upon necropsy it was determined that a gavage error had occurred. One male, D-4089, from group 4 was moved to group 5 prior to dosing group 5 with a radiolabeled dose on day 28 so as to increase the number of male animals in group 5.

20.2 Clinical Observations

The incidence of clinical observations is summarized in Table 1. Individual clinical examination data are presented in Appendix A. There were no significant clinical signs associated with treatment.

20.3 Body Weight

Summaries of weekly mean body weights are shown in Table 1. Mean body weight gain summaries are shown in Table 2. Individual animal body weight data and individual animal body weight gain data can be found in Appendices B and C, respectively.

The mean body weights at day 28 for male rats in the treated groups were lower than control values. The decrease in mean body weight at day 28 was notably lower in the male animals receiving 100 and 1000 mg HMDS/kg body weight. The percentage of weight gain in groups 3 and 4 was only ~ 7 and 6% as compared to male control animals where the percentage weight gain was ~15%.

20.4 Morphological Pathology

Macroscopic Examination:

Macroscopic pathology data are presented in a contributing scientist report in Appendix D. There were no gross morphological changes attributable to the test article. Findings were generally typical of rats of this age, stock, and test article administration method, but unrelated to test article dosage.

Microscopic Examination:

The incidences of microscopic pathology findings are given in a Contributing Scientist Report in Appendix D. In the group administered HMDS at 10 mg/kg/day for 28 days, the amount and histomorphology of protein droplet accumulation in the cortex did not appear different from controls. A very subtle change was observed in the group receiving 100 mg/kg/day. The only indication of hyaline droplet nephropathy was the occasional angular cytoplasmic inclusion in the P2 proximal tubule segments where alpha 2u protein is normally visible and resorbed. The nephropathy in this group was graded as minimal. Alpha 2u nephropathy was graded as moderate in all sections examined of group 4, 1000 mg/kg/day, animals. The contributing scientists report states the following. At 1000 mg/kg/day there was moderate multifocal tubular basophilia in all sections, indicating a substantial reparative process. In addition to the increase in multifocal tubular basophilia, the nephropathy was characterized by an increase in the portion of the cortex with tubular luminal protein droplets and epithelial cytoplasmic inclusions. Most of the latter had angular crystalline shapes. Also, there were one to several large eosinophilic granular casts at the cortico-medullary junction in all sections. These represent accumulated dead cortical tubule epithelial cells and are typical of this nephropathy. Scattered focal mineralization of the outer medulla was also observed in some cases.

20.5 Group 5: Concentrations and Body Burden of HMDS and Total Radioactivity in Blood and Kidney

Radioactivity Administered per Animal:

Total radioactivity administered to female and male rats was an average of 114 and 166 uCi per animal, respectively.

Blood Concentrations:

The mean measured concentrations of parent and total radioactivity in blood were similar between group 5 males and females. Males were found to have in blood an average of 13.5 ug HMDS/g blood and total radioactivity of 49.2 ug equivalents HMDS/g blood. In female blood, concentrations determined were 15.0 ug HMDS/g and total radioactivity of 48.5 ug equivalents HMDS/g (Table 4; details in Appendix E).

From the PBPK model described by Dobrev *et al.*, 2003 the predicted blood concentrations of HMDS (ug/g blood) was 25 ug/g blood at 2 h post-dosing. This predicted value is ~1.6 times greater than the actual measured blood concentrations.

Kidney Concentrations:

The mean measured concentrations of parent and total radioactivity in kidney (one kidney analyzed from each animal, group 5) were different between males and females (Table 4; details in Appendix E). Males were found to have in kidney an average of 120 ug HMDS/g kidney and total radioactivity of 232 ug equivalents HMDS/g. In female kidney, average concentrations determined were 58.7 ug HMDS/g and total radioactivity of 121 ug equivalents HMDS/g. Two-fold higher concentrations of parent material and total radioactivity were found in kidney of male vs. female animals. However, the ratio of parent to total radioactivity for males and females in group 5 was approximately 50%.

Blood and Kidney Radioactive Burden:

The percentage of radioactivity in the blood of male and female animals that represents metabolites is approximately 70%. In kidney of male and female animals the percentage of radioactivity that represents metabolites is approximately 50% (Table 4).

20.6 Group 5: Reversible Binding of HMDS/Metabolites to Alpha 2u-globulin

Presence of Alpha 2u-globulin in Male Rat Kidney:

The peak region in which alpha 2u-globulin protein elutes was resolved from most other proteins in rat kidney cytosol by HPLC using a Hydrophobic Interaction Chromatography (HIC) column. UV detection, diode array detector (DAD), was used to visualize the protein separation from male and female rat kidney cytosol (Figures 1 and 2, respectively). On average, the peak region in which alpha 2u-globulin elutes was observed to be 27% of the total peak area of the male kidney samples while the integrated percent peak area for female kidney samples was 10% (Tables 5 and 6; details in Appendix F). A qualitative dot blot technique was used to confirm the presence of alpha 2u-globulin in the eluent peak fraction. In this technique, protein fractions eluted from the HIC column were applied to a nitrocellulose membrane followed by immunodetection with purified anti-rat alpha 2u-globulin goat IgG (Figure 3). Chymotrypsinogen A and ribonuclease A were utilized as negative controls to demonstrate that non-specific protein binding did not occur. Alpha 2u-globulin was present in the male rat kidney eluent HIC fraction and not in the female rat kidney eluent HIC fraction.

Radioactivity Co-eluting with the Alpha 2u-globulin Peak:

Radioactivity co-eluting with the alpha 2u-globulin peak region was on average 54% of the total radioactivity per male kidney sample (Table 6; details in Appendix F). In addition to this peak, another radioactive species was observed that was either associated with earlier eluting proteins, ~ 3 minutes (Figure 4) or a free unbound aqueous soluble metabolite. Female rat kidney samples demonstrated no co-elution at the later retention time where alpha 2u-globulin elutes, however, the early eluting radioactive species was observed at low levels (Figure 5).

Reversible Binding of HMDS/Metabolites to Alpha 2u-globulin, Competition with D-Limonene Oxide:

The alpha 2u-globulin peak region remained, on average, as approximately 26 and 28% of the total peak area through incubation with negative control (DMSO) and D-limonene oxide treatment, respectively (Table 6, Figures 6 and 7). Due to its known affinity to alpha 2u-globulin, D-Limonene-oxide was incubated with kidney cytosol samples to displace bound ¹⁴C-HMDS and/or metabolite to the protein to indicate reversible binding.

The D-limonene treated samples demonstrated complete loss of bound radioactivity at the same elution time as the alpha 2u-globulin peak region and the appearance of an additional radioactive peak at ~ 4 minutes (Figures 8 and 9). D-Limonene oxide was able to displace bound ¹⁴C-HMDS and/or metabolites to the alpha 2u-globulin protein and thus demonstrate that reversible binding of HMDS and/or metabolites occurred in male kidney samples.

21 DISCUSSION

HMDS is non-genotoxic and acute oral, inhalation and dermal toxicity studies demonstrated a low level of toxicity following administration/exposure of rats to HMDS. In a 28-day oral gavage study with Sprague-Dawley rats, increased protein droplets in male rat kidney were observed at a dose of 1000 mg/kg bw (Crofoot *et al.*, 1990). In a 2-week inhalation study, increased protein droplets were observed in male rat kidney at 500 and 1000 ppm. The effects of increased tubular regeneration, hyaline and granular casts, tubular and papillary mineralization, and fibrosis were observed in the male rat kidney following 90 days of inhalation exposure to concentrations greater or equal to 1500 ppm (Cassidy *et al.*, 2001).

Following a nose-only exposure for 6 h/day for 6 days at 0 or 5000 ppm of HMDS to male F344 rats, alpha 2u-globulin was identified in kidney sections by immunohistochemical staining and HMDS exposure clearly increased the amount and altered the morphology of alpha 2u-globulin stained material (Crissman *et al.*, 2002).

In a 2-year chronic carcinogenicity study with HMDS by inhalation exposure, a principal toxicological effect was an increase in kidney tumors in male rats at the two highest exposure concentrations; 1600 and 5000 ppm (Dotti *et al.*, 2004; Jovanovic *et al.*, 2005). At the one year interim sacrifice group of animals, kidney lesions consistent with alpha 2u-globulin nephropathy were observed in male rats that were exposed to concentrations of 1600 and 5000 ppm HMDS.

In summary, standard histopathology examination over several studies demonstrated primary lesions and tumor sequelae consistent with alpha 2u-globulin nephropathy.

The mechanism of alpha 2u-globulin nephropathy and renal carcinogenesis has been studied extensively with various chemicals that elicit this nephropathy and examples of these studies can be found in Borghoff *et al.*, 1990 and 1993. Alpha 2u-globulin

mediated nephrotoxicity is specific to male rats and is associated with renal accumulation of alpha 2u-globulin, a low molecular weight protein synthesized and secreted from the liver of male rats.

Chemically induced renal tumors in male rats occurring via the alpha 2u-globulin mediated nephropathy are not used in the hazard characterization process to assess human risk since this mechanism of renal tumor formation is specific to the male rat (EPA, 1991). Therefore, studies that demonstrate this mechanism for renal tumors observed in the 2-year chronic carcinogenicity study with HMDS will aid in the risk assessment process for HMDS.

EPA criteria (1991) for confirming the alpha 2u-globulin mechanism for renal tumors are; an increased number and size of hyaline droplets in renal proximal tubule cells of treated male rats, confirmation that the accumulating protein in hyaline droplets is indeed alpha 2u-globulin, and demonstration of the pathological sequence of lesions associated with alpha 2u-globulin. Confidence in the mechanism is increased with the following; a negative mouse bioassay, demonstration of reversible binding of a chemical to alpha 2u-globulin, reduced lysosomal degradation of the alpha 2u-globulin complex, retention of the chemical in the male rat kidney, increased cell proliferation in the kidney and known structure activity relationships.

IARC criteria (1998) to demonstrate the alpha 2u-globulin mechanism are the following; the chemical is non-genotoxic, demonstration of male rat specific nephropathy and tumorigenicity, observed protein droplet accumulation in the kidney and the characteristic progression of this nephropathy, positive identification of the accumulating protein as alpha 2u-globulin, demonstration of reversible binding of alpha 2u-globulin to the chemical, increase in cortical cell proliferation and a similar dose response for nephropathy and tumors.

The toxicity studies described above with HMDS fulfill the three major EPA criteria for an alpha 2u-globulin mediated mechanism of renal kidney tumors in male rats. Confidence in this mechanism is increased with data from the study of Durham *et al.*, 2006. Data from this inhalation study supports the observations in previous studies with HMDS where an alpha 2u-globulin mechanism for renal toxicity in male rats has been noted. The percentage of total radioactivity in kidney as parent HMDS, increased from ~39% to 62% following the single and repeated exposures, respectively. The ratio of

parent AUC in kidney to parent AUC in blood was ~30 fold higher than the same ratio calculated for liver tissue. This observation is consistent with the hypothesis that HMDS is an alpha 2u-globulin ligand and with a previous observation of increased alpha 2u-globulin in the kidney after exposure to HMDS.

The HMDS studies described above also fulfill all but one of the criteria given by IARC for an alpha 2u-globulin mediated mechanism for renal tumors in the male rat. Demonstration of the reversible binding of HMDS to the protein is one of the criteria that was not met in IARC's list of criteria. Also confidence by EPA in this mechanism is increased if reversible binding or non-covalent bonding is demonstrated since the formation of a chemical-alpha 2u complex is critical to the development of male rat specific nephropathy and tumor formation.

A previously conducted study was undertaken to evaluate the reversible binding of alpha 2u-globulin to HMDS. Male and female F344 rats were exposed by inhalation for 6 days to HMDS at 5000 ppm (HES Study Number 10120). Established direct and indirect approaches that demonstrate a chemical-alpha 2u-globulin interaction such as gel filtration, DEAE anion exchange chromatography and headspace gas chromatography (Borghoff *et al.*, 1993, Prescott-Matthews *et al.*, 1999 and Williams and Borghoff 2001) were unsuccessful in characterizing binding between HMDS/metabolites with alpha 2u-globulin most likely due to the extreme hydrophobic and volatile nature of HMDS.

A new approach using Hydrophobic Interaction Chromatography (HIC) was developed to isolate the alpha 2u-globulin protein peak region. A dot blot using commercially available polyclonal antibodies raised against purified rat alpha 2u-urinary globulin was used to positively identify the presence of alpha 2u-globulin in the protein fraction eluted from the HIC column. Radioactivity co-eluting with the alpha 2u-globulin peak region was then monitored to determine the binding of HMDS or metabolite to the protein.

This new approach was developed using kidneys from male and female rats that were administered radiolabeled HMDS by oral gavage in a corn oil solution at a dose level of 100 mg/kg; ~100 uCi/animal. It was determined that with administration of ¹⁴C-HMDS by oral gavage, ~ 10-fold more radioactivity per g of kidney tissue was detected vs. exposure by inhalation although the ug equivalents of HMDS per g kidney was ~6-fold higher following inhalation exposure. The specific activity of the solution used to achieve the inhalation exposure was 0.053 mCi/g HMDS whereas the specific activity of

the dosing solution used for oral gavage administration was 0.228 mCi/g HMDS. To investigate reversible binding in the present study a dose level of 1000 mg/kg bw and ~200 uCi/animal was used.

The present study has demonstrated that the characteristics of alpha 2u-globulin nephropathy increased with increasing dose of HMDS administered to male rats by oral administration for 28 days. It was also observed that the radioactive burden was greater in male kidney than in female kidney. The presence of alpha 2u-globulin in male rat kidney cytosol was confirmed by an immunodetection dot blot procedure and this protein was not present in cytosol derived from female rat kidney. The reversible binding of HMDS/metabolites to the protein in male rat kidney was demonstrated by incubation of male rat kidney cytosol from males treated with HMDS with D-limonene oxide since D-limonene oxide displaced HMDS/metabolites from the protein. Demonstration of a non-covalent chemical-alpha 2u-globulin complex meets an important criterion in establishing the rat specific alpha 2u-globulin mechanism of toxicity for renal tumors, a mechanism that is not relevant to humans.

22 CONCLUSIONS

Demonstration of reversible binding of HMDS or metabolites to alpha 2u-globulin in male rat kidney samples from HMDS treated rats, along with the present and previous observations of increased hyaline droplet formation, male rat specific kidney tumors, and the presence of alpha 2u-globulin in kidney sections detected by immunohistochemistry shows that this species specific mechanism, alpha 2u-nephropathy, of toxicity is responsible for the rat kidney neoplasia observed in a previous chronic/carcinogenicity study with HMDS. Therefore, this mechanism of kidney tumor formation in male rats following administration of HMDS is species specific and not relevant to human cancer risk assessment.

23 ARCHIVE

Protocol, amendments and deviations as applicable, study authorization documentation, raw data, correspondence, samples/specimens as applicable, and final report (at minimum) will be retained in the HES Archives, Dow Corning Corporation, 2200 W. Salzburg Road, Auburn, MI. 48611.

REFERENCES

- Borghoff, S.J., and Lagarde, W.H (1993). Assessment of binding of 2,4,4,-trimethyl-2-pentanol to low-molecular-weight proteins isolated from kidneys of male rats and humans. *Toxicol. Appl. Pharmacol.* **119**: 228-235.
- Borghoff, S.J., Short, B.G., and Swenberg, J.A. (1990). Biochemical Mechanisms and pathobiology of α 2u-globulin nephropathy. *Annu. Rev. Pharmacol. Toxicol.* **30**: 349-367.
- Cassidy, S. L., Dotti, A., Kolesar, G. B., Dochterman, L. W., Meeks, R. G. and Chevalier, H. J. (2001). Hexamethyldisiloxane: A 13-week subchronic whole-body vapor inhalation toxicity study in F344 rats. *Int. J. Toxicol.* **20**(6): 391-399.
- Crofoot, S.D., Stanton, E., Siddiqui, W.H. and Zimmer, M.A. (1990). A 28-Day Subchronic Oral Gavage Feasibility Study of Various Low Molecular Weight Silicone Oligomers in Rats. Report of Dow Corning Corporation (1990-I0000-35105).
- Crissman, J.W., Crofoot, S.D. and Regan, J.M. (2002). Non-Regulated Study: A One-Week Vapor Inhalation Study to Evaluate by Immunohistochemistry the Effect of Hexamethyldisiloxane (HMDS) on Alpha 2u-Globulin Accumulation in the Kidneys of Male Fisher 344 Rats. Report of Dow Corning Corporation (2002-I000-51723).
- Dobrev, I. D., Reddy, M. B., Plotzke, K. P., Varaprath, S., McNett, D. A. and Andersen, M. E. (2003). Closed-chamber inhalation pharmacokinetic studies with hexamethyldisiloxane in the rat. *Inhalation Toxicol.* **15**: 589-617.
- Domoradzki, J. Y., Thackery, L. M. and McNett, D. A. (2007, report in preparation). Inhalation (nose-only) reversible binding study in male and female F344 rats and a limited disposition PK study in female rats. HES Study Number: 10120-101.
- Dotti, A., Smith, P.A., Chevalier, H.J. and Crofoot, S.D. (2004). Hexamethyldisiloxane: A 24-Month Combined Chronic Toxicity Study and Oncogenicity Whole Body Vapor Inhalation Study in Fisher 344 Rats. Report of Dow Corning Corporation (2004-I0000-53896).
- Durham, J. A., McNett, D. A., Tobin, J. M., Domoradzki, J. Y. and Plotzke, K. P. (2006). Disposition of ^{14}C -hexamethyldisiloxane (^{14}C -HMDS) in male Fischer 344 rats following single and repeated inhalation exposure. *The Toxicologist*.
- Jovanovic, M. L., Crofoot, S. D., Crissman, J. W., Plotzke, K. P., Meeks, R. H. (2005). Chronic toxicity and oncogenicity study of hexamethyldisiloxane in Fischer 344 rats. *The Toxicologist*.

Lehman-McKeeman, L.D., Rodriguez, P.A., Takigiku, R., Caudill, D., and Fey, M.L. (1989). D-Limonene-induced male rat specific nephrotoxicity: Evaluation of the association between d-limonene and α 2u-globulin. *Toxicol. Appl. Pharmacol.* **91**: 182-192.

National Research Council, Guide for the Care and Use of Laboratory Animals, *National Academy Press, Washington, D.C.*, **32**, 1996.

Poet, T.S. and Borghoff, S.J. (1997). *In vitro* uptake of methyl tert-butyl ether in male rat kidney: use of a two compartment model to describe protein interactions. *Toxicol. Appl. Pharmacol.* **145**: 340-348.

Prescott-Mathews, J.S., Poet, T.S., and Borghoff, S.J. (1999). Evaluation of the *in vivo* interaction of methyl tert-butyl ether with α 2u-globulin in male F-344 rats. *Toxicol. Appl. Pharmacol.* **157**, 60-67.

Swenberg, J.S. (1993). α 2u-Globulin nephropathy: Review of the cellular and molecular mechanisms involved and their implications for human risk assessment. *Environ. Health Perspect.* **101** (suppl. 6), 39-44.

U.S. Department of Agriculture, Animal Welfare Act, 9 CFR Subchapter A, Parts 1, 2 and 3.

Varaprath, S., McMahon, J. M., and Plotzke, K.P. (2003). Metabolites of hexamethyldisiloxane and decamethylcyclopentasiloxane in Ficsher 344 rat urine – A comparison of a linear and a cyclic siloxane. *Drug Metabolism and Disposition.* **31**: 206-214.

Williams, T.M., and Borghoff, S.J. (2001). Characterization of tert-butyl alcohol binding to α 2u-globulin in F-344 rats. *Toxicological Sciences.* **62**: 228-235.

Table 1 – Summary of Clinical Observations

Group Sex	Clinical Sign	Severity	Day numbers relative to Start Date													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1m	ANIMALS ALIVE	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	ANIMALS NORMAL	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	Soiling
	TOTAL

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28
Group 3 - 100 mg HMDS/kg

Table 1 – Summary of Clinical Observations (cont.)

Group Sex	Clinical Sign	Severity	Day numbers relative to Start Date													
			1	1	1	1	1	1	2	2	2	2	2	2		
1m	ANIMALS ALIVE	5	5	6	7	8	9	0	1	2	3	4	5	6	7	8
	ANIMALS NORMAL	4	4	5	5	5	5	5	5	5	5	5	5	5	5	5
	Soiling	1	1
	TOTAL	1	1
	Removed from study to be used for control blood collection.	1
	TOTAL	1

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Table 1 - Summary of Clinical Observations (cont.)

Group Sex	Clinical Sign	Day numbers relative to Start Date													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
2m	ANIMALS ALIVE	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	ANIMALS NORMAL	4	4	4	5	4	4	5	5	5	5	5	5	5	4
	Soiling	1	1	1	.	1	1	1
	TOTAL	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28
Group 3 - 100 mg HMDS/kg

Table 1 – Summary of Clinical Observations (cont.)

Group Sex	Clinical Sign	Severity	Day numbers relative to Start Date																		
			1	2	3	4	5	6	7	8	9	10									
3m	ANIMALS ALIVE	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
	ANIMALS NORMAL	3	3	4	4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	Injury Apparent Mechanical	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	Excessive Hair Loss Limbs	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	Soiling
	Present TOTAL
	Red TOTAL	1	1

Nominal Dose: Group 1 - 0 mg HMDS/kg Group 2 - 10 mg HMDS/kg Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Table 1 - Summary of Clinical Observations (cont.)

Group Sex	Clinical Sign	Day numbers relative to Start Date													
		1	2	3	4	5	6	7	8	9	10				
5m	ANIMALS ALIVE	10	10	10	10	10	10	10	10	10	10	10	10	10	
	ANIMALS NORMAL	6	7	7	8	3	4	3	5	6	6	4	4	5	5
	Injury Apparent Mechanical	2	2	2	2	2	2	2	2	2	2
	Broken/Missing Toe Nail	2	2	2	2	2	2	2	2	2	2
	TOTAL	3	1	1	2	7	6	7	4	4	3	5	4	3	4
	Soiling	1	2	2
	Dark Yellow	4	3	3	2	7	6	7	4	4	3	5	4	3	4
	TOTAL	10	10	10	10	10	10	10	10	10	10	10	10	10	10
5f	ANIMALS ALIVE	8	9	9	10	9	8	7	5	5	9	10	10	10	10
	ANIMALS NORMAL
	Eye Lid(s) partially closed
	Present
	TOTAL	2	1	1	1	1	2	3	5	5	1	4	1	1	1
	Soiling
	Clear	1
	Red	2	.	.	.	1	2	2	5	5	1	.	4	1	.
	Yellow	.	1	1
	TOTAL	2	1	1	1	1	2	3	5	5	1	4	1	1	1

Nominal Dose: Group 1 - 0 mg HMDS/kg Group 2 - 10 mg HMDS/kg Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Table 1 -- Summary of Clinical Observations (cont.)

Group Sex	Clinical Sign	Day numbers relative to Start Date													
		1	1	1	1	1	2	2	2	2	2	2			
5m	ANIMALS ALIVE	1	6	7	8	9	0	1	2	3	4	5	6	7	8
	ANIMALS NORMAL	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	Injury Apparent Mechanical	3	5	5	8	7	9	8	7	8	7	8	7	8	7
	Broken/Missing Toe Nail	2	2	2	2	1	1	1	1
	TOTAL	2	2	2	2	1	1	1	1
	Soiling	6	4	4	.	2	1	2	3	2	3	2	1	2	3
	Dark Yellow
	TOTAL	6	4	4	.	2	1	2	3	2	3	2	1	2	3
	ANIMALS ALIVE	10	10	10	10	10	10	10	10	10	10	10	10	10	10
5f	ANIMALS NORMAL	7	5	5	7	6	5	7	7	8	7	10	8	5	4
	Eye Lid(s) partially closed
	TOTAL
	Soiling	1
	Clear	3	5	5	3	4	5	3	2	2	3	.	2	5	6
	Red
	Yellow	3	5	5	3	4	5	3	3	2	3	.	2	5	6
	TOTAL	3	5	5	3	4	5	3	3	2	3	.	2	5	6

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg

Group 2 - 10 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Group 3 - 100 mg HMDS/kg
Group 3 - 100 mg HMDS/kg

Table 2 - Summary of Weekly Mean Body Weights (grams)

Group Sex	Day numbers relative to Start Date						
	1	8	15	22	28		
1m	244.70	251.84	261.48	271.86	280.52		
Mean	5.09	4.97	5.80	6.60	6.45		
S.D.	5	5	5	5	5		
N							
2m	243.62	249.24	256.86	268.02	274.26		
Mean	6.31	9.09	8.56	10.14	10.66		
S.D.	5	5	5	5	5		
N							
3m	245.68	252.18	259.50	269.02	275.06		
Mean	7.02	6.68	8.08	7.58	8.70		
S.D.	5	5	5	5	5		
N							
4m	245.04	243.66	249.00	257.38	262.28		
Mean	6.54	4.82	3.08	4.24	6.80		
S.D.	5	5	4	4	4		
N							
5m	242.56	241.56	247.11	252.22	257.33		
Mean	6.90	8.54	8.25	8.87	9.69		
S.D.	10	10	10	10	10		
N							
5f	162.67	163.57	166.42	169.99	171.36		
Mean	2.32	3.75	4.43	5.16	6.09		
S.D.	10	10	10	10	10		
N							

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg

Group 2 - 10 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Group 3 - 100 mg HMDS/kg

Table 3 - Summary of Weekly Mean Body Weight Gains (grams)

Group Sex	Base Weight Day	From: To:	Day numbers relative to Start Date						% Gain
			1 8	8 15	15 22	22 28	28 1	1 28	
1m	244.70	Mean	7.14	9.64	10.38	8.66	35.82	14.68	
	5.09	S.D.	3.42	2.90	3.07	2.69	8.06	3.53	
	5	N	5	5	5	5	5	5	
2m	243.62	Mean	5.62	7.62	11.16	6.24	30.64	12.55	
	6.31	S.D.	3.43	1.45	3.42	2.00	4.90	1.74	
	5	N	5	5	5	5	5	5	
3m	245.68	Mean	6.50	7.32	9.52	6.04	29.38	11.96	
	7.02	S.D.	4.26	1.72	1.49	1.67	3.91	1.54	
	5	N	5	5	5	5	5	5	
4m	245.04	Mean	-1.38	3.75	8.38	4.90	16.10	6.60	
	6.54	S.D.	3.23	2.89	1.86	2.80	9.04	3.80	
	5	N	5	4	4	4	4	4	
5m	242.56	Mean	-1.00	5.55	5.11	5.11	14.77	6.07	
	6.90	S.D.	3.31	3.13	3.29	2.19	3.89	1.48	
	10	N	10	10	10	10	10	10	
5f	162.67	Mean	0.90	2.85	3.57	1.37	8.69	5.33	
	2.32	S.D.	2.04	2.50	2.13	1.64	4.59	2.79	
	10	N	10	10	10	10	10	10	

Abs Gain = absolute bodyweight gain between base period and end of the analysis period
% Gain = percentage bodyweight gain between base period and end of the analysis period

Nominal Dose: Group 1 - 0 mg HMDS/kg Group 2 - 10 mg HMDS/kg Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Table 4 – Concentration of Parent and Radioactivity and Percentage of Parent/Radioactivity at 2 h Post-Dosing

Sample ID	Blood			Kidney		
	Parent µg/g	Total Rad µg eq/g	Parent as % of Total	Parent µg/g	Total Rad µg eq/g	Parent as % of Total
4094	13.9	52.1	26.6%	137	266	51.6%
4095	16.1	49.0	32.7%	146	250	58.2%
4096	13.1	49.1	26.7%	127	250	50.7%
4097	16.6	50.8	32.6%	137	260	52.6%
4098	11.7	48.2	24.2%	111	220	50.4%
4089	12.5	42.2	29.5%	118	208	56.7%
4100	14.3	54.9	26.0%	123	267	45.8%
4101	6.88	44.1	15.6%	80.8	172	47.1%
4102	11.0	40.6	27.1%	93.8	165	56.9%
4103	18.9	60.7	31.2%	131	261	50.3%
Average	13.5	49.2	27.2%	120	232	52.0%

Sample ID	Blood			Kidney		
	Parent µg/g	Total Rad µg eq/g	Parent as % of Total	Parent µg/g	Total Rad µg eq/g	Parent as % of Total
4104	11.7	39.3	29.8%	39.2	85.3	45.9%
4105	14.0	46.8	30.0%	52.7	110	47.8%
4106	16.0	48.4	33.1%	64.1	127	50.5%
4107	15.1	49.5	30.6%	56.8	115	49.2%
4108	13.0	42.0	30.9%	46.0	89.2	51.6%
4109	16.2	50.6	32.1%	65.9	150	44.0%
4110	12.5	49.9	25.1%	58.4	134	43.7%
4111	17.8	52.3	34.1%	66.8	122	54.7%
4112	17.3	60.2	28.7%	70.1	154	45.4%
4113	16.4	46.1	35.6%	66.9	119	56.3%
Average	15.0	48.5	31.0%	58.7	121	48.9%

Sample ID 4094 - 4103 (including 4089) are male Fischer 344 rats.
Sample ID 4104 - 4113 are female Fischer 344 rats.

Table 5 – Percent Peak Area Summary of the Alpha 2u-globulin Peak Region from HIC HPLC/DAD Analysis

Analysis Date	Sample ID Males	% Peak Area	Control % Peak Area	D-limox treated % Peak Area	Sample ID Females	% Peak Area	Control % Peak Area	D-limox treated % Peak Area
6/21/2007	4094	32.45*	25.52	26.81	4104	9.46	7.99	7.86
6/21/2007	4095	23.47	23.91	25.49	4105	8.52	7.51	7.38
7/2/2007	4096	26.16	25.47	26.55	4106	9.48	9.37	8.76
7/2/2007	4097	28.49	28.73	30.50	4107	9.41	9.61	9.29
7/2/2007	4098	27.14	26.54	27.81	4108	8.51	8.67	8.21
7/6/2007	4089	31.56	27.97	28.70	4109	14.04	NA**	NA
7/6/2007	4100	27.75	24.14	25.70	4110	10.36	NA	NA
7/6/2007	4101	29.17	27.03	27.80	4111	11.10	NA	NA
7/6/2007	4102	26.31	24.78	26.59	4112	10.63	NA	NA
7/6/2007	4103	26.60	24.42	29.09	4113	9.93	NA	NA
Average		27.41	25.85	27.50		10.14	8.63	8.30

*injection volume double that of other samples (excluded from avg)

**NA - not analyzed

Table 6 - Percent Peak Area Summary of ¹⁴C-HMDS and/or Metabolites from HIC HPLC/RAD Analysis

Analysis Date	Sample ID Males	% Peak Area	DMSO Control		D-limonin treated % Peak Area
			% Peak Area	% Peak Area	
6/21/2007	4094	56.58	61.16	13.5*	
6/21/2007	4095	59.43	52.99	0.00	
7/2/2007	4096	50.64	48.75	0.00	
7/2/2007	4097	60.84	52.92	0.00	
7/2/2007	4098	54.18	48.96	0.00	
7/6/2007	4089	53.18	58.76	0.00	
7/6/2007	4100	44.43	35.45	0.00	
7/6/2007	4101	53.49	47.38	0.00	
7/6/2007	4102	56.68	45.92	0.00	
7/6/2007	4103	48.41	44.12	0.00	
Average		53.79	49.64	0.00	

*excluded from average calculation as integrated peak area appears to be baseline noise

Figure 1 - Representative Diode Array Detector (DAD) Chromatogram of Male Kidney Cytosol Profile

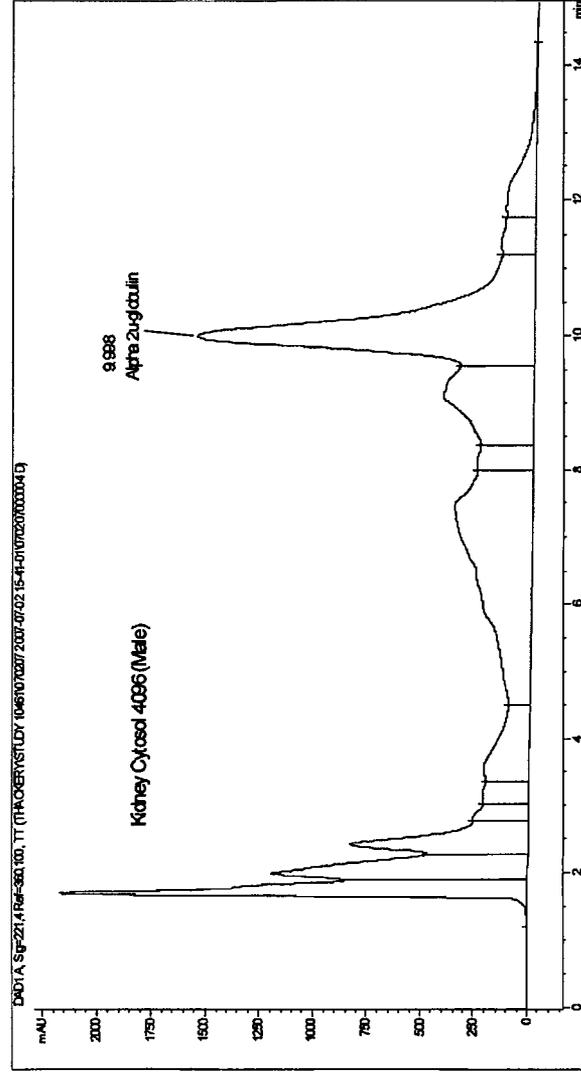


Figure 2 - Representative Diode Array Detector (DAD) Chromatogram of Female Kidney Cytosol Profile

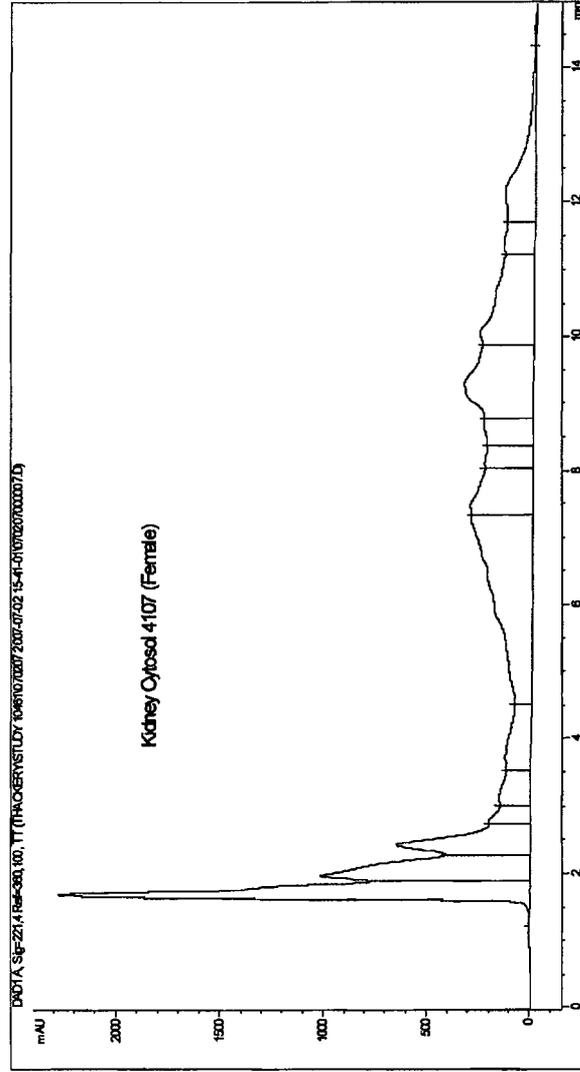


Figure 3 - Dot Blot of Representative Male versus Female Kidney Cytosol Fractions Collected From Hydrophobic Interaction Chromatography (HIC)

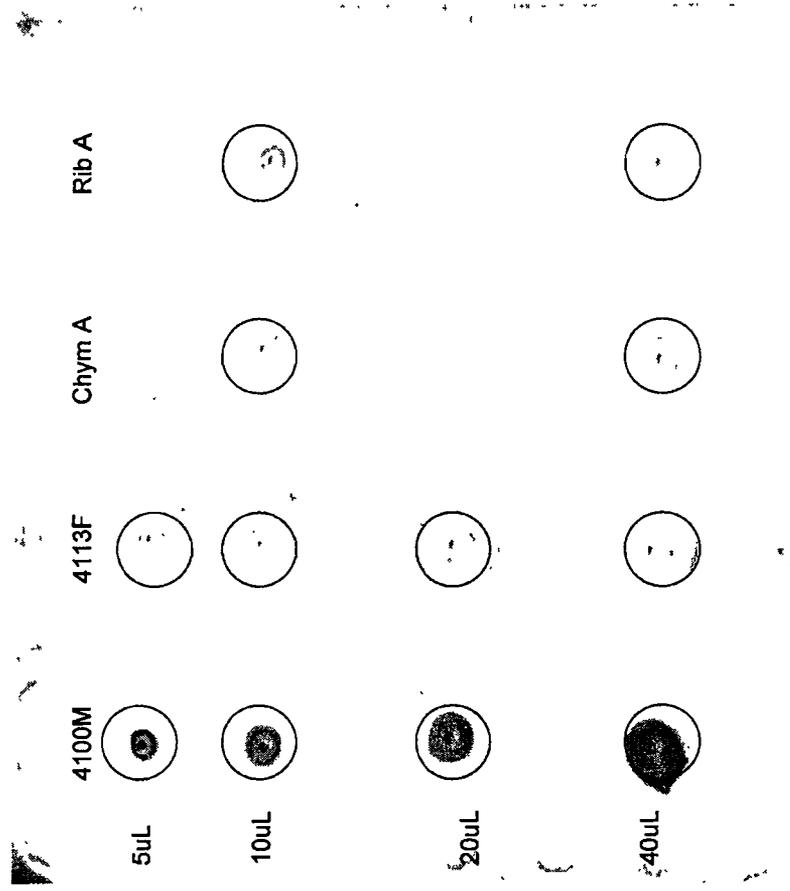


Figure 4 - Representative Radiometric Detector (RAD) Chromatogram of Male Kidney Cytosol Profile

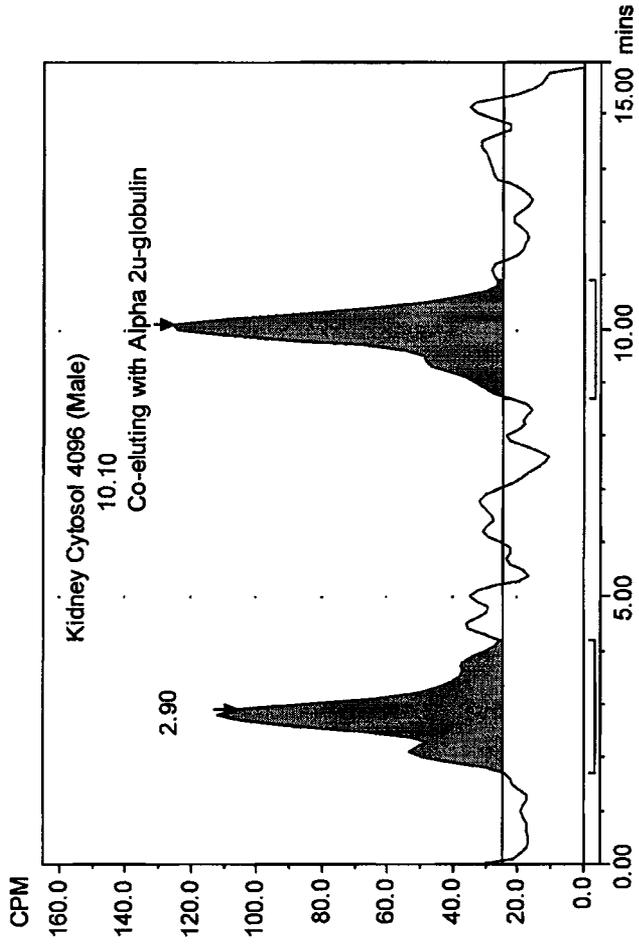


Figure 5 - Representative Radiometric Detector (RAD) Chromatogram of Female Kidney Cytosol Profile

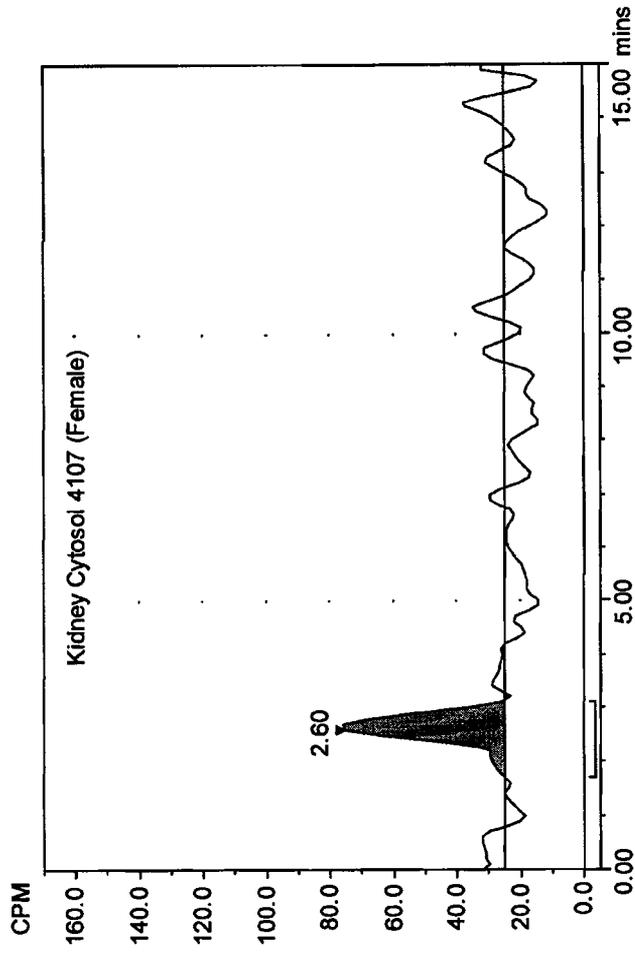


Figure 6 - Representative Diode Array Dectector (DAD) Chromatogram of Male Kidney Cytosol Profile after Incubation with DMSO (Negative Control)

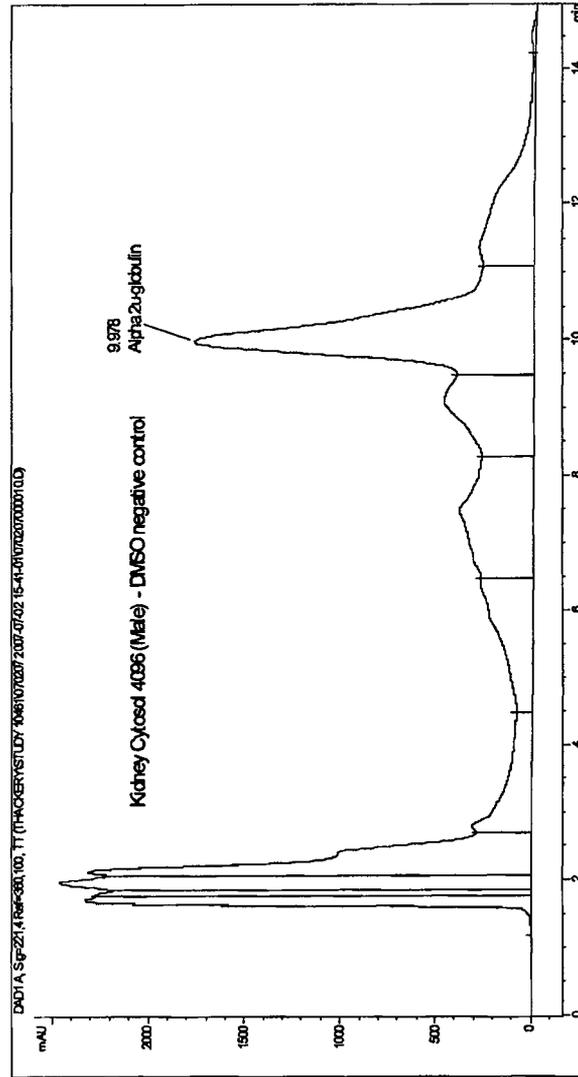


Figure 7 - Representative Diode Array Dectector (DAD) Chromatogram of Male Kidney Cytosol Profile after Incubation with D-Limonene Oxide

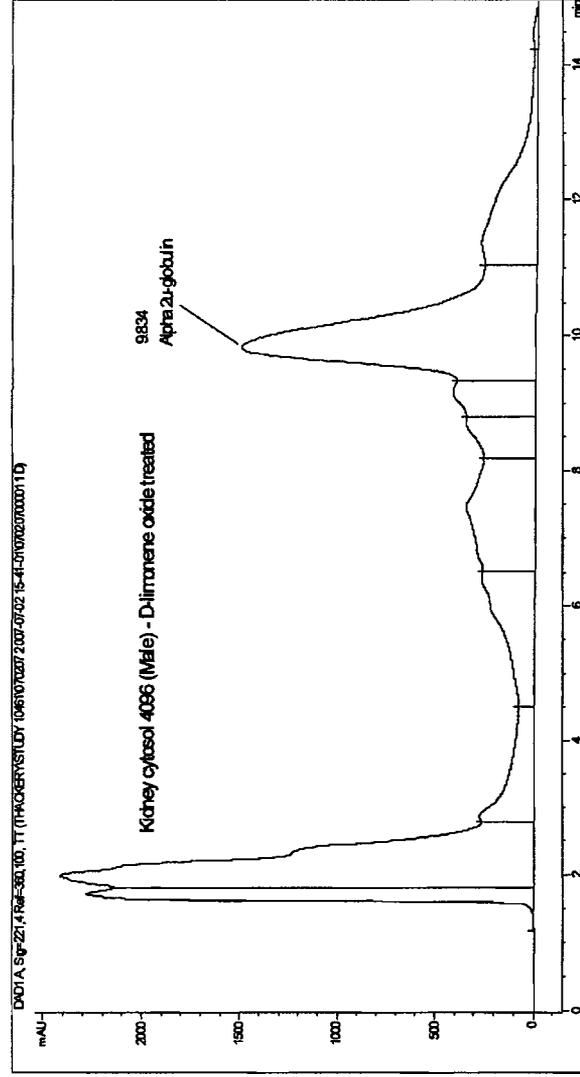


Figure 8 - Representative Radiometric Detector (RAD) Chromatogram of Male Kidney Cytosol Profile after Incubation with DMSO (Negative Control)

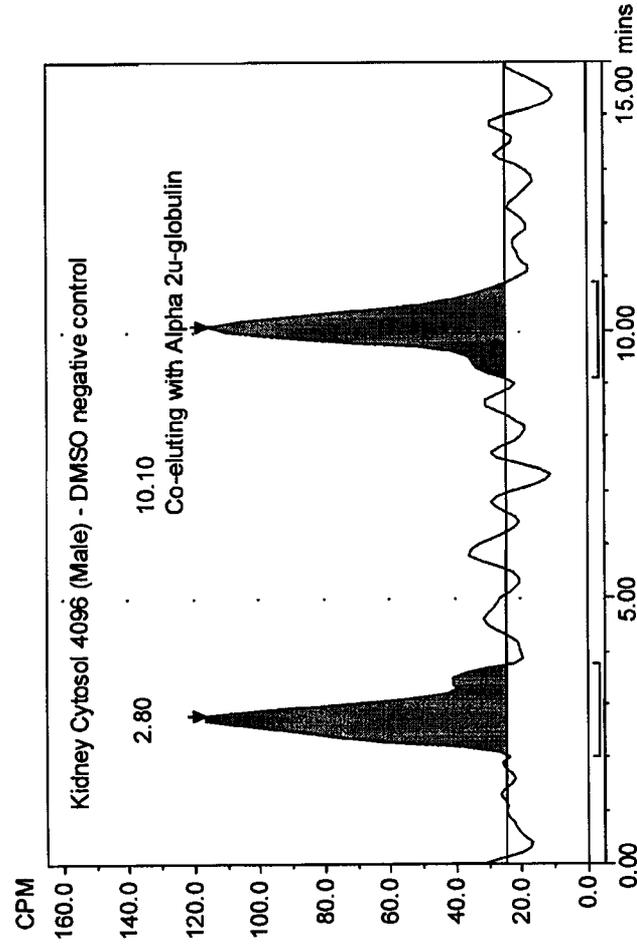
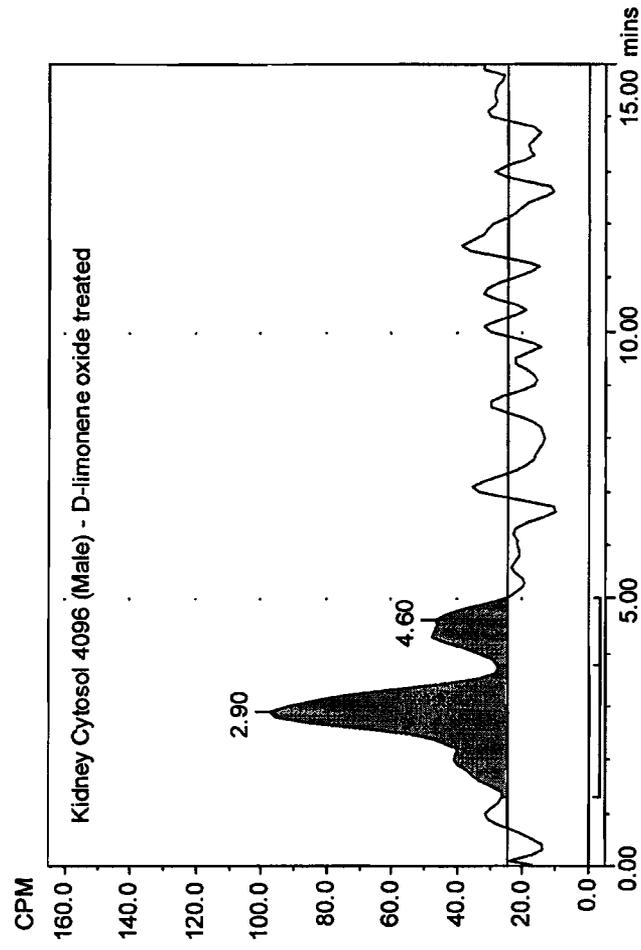


Figure 9 - Representative Radiometric Detector (RAD) Chromatogram of Male Kidney Cytosol Profile After Incubation with D-Limonene Oxide



Appendix A -- Individual Clinical Observations

Group Sex	Animal Number	Clinical Sign	Site	Day numbers relative to Start Date																
				1	2	3	4	5	6	7	8	9	0	1	2	3	4			
1m	4074	No Abnormalities Detected	Nose	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	4075	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	4076	No Abnormalities Detected	Nose
	4077	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	4078	No Abnormalities Detected	

X = Present
D = Dark Yellow
C = Clear
B = Broken/Missing Toe Nail
R = Red
Y = Yellow

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Appendix A - Individual Clinical Observations (cont.)

Group Sex	Animal Number	Clinical Sign	Site	Day numbers relative to Start Date														
				1	2	3	4	5	6	7	8	9	0	1	2	3	4	
2m	4079	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	4080	No Abnormalities Detected		.	X	.	X	X	X	X	X	X	X	X	X	X	X	X
	4081	No Abnormalities Detected	Nose	R	R	R	R	.	X	X	X	X	X	X	X	X	X	X
	4082	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	4083	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

X = Present
D = Dark Yellow
C = Clear
B = Broken/Missing Toe Nail
R = Red
Y = Yellow

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Appendix A – Individual Clinical Observations (cont.)

Group Sex	Animal Number	Clinical Sign	Site	Day numbers relative to Start Date																				
				1	5	6	1	7	8	9	1	2	0	1	2	2	3	4	2	2	5	6	2	2
2m	4079	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	4080	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	4081	No Abnormalities Detected	Nose
	4082	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	4083	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

X = Present
D = Dark Yellow
C = Clear
B = Broken/Missing Toe Nail
R = Red
Y = Yellow

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Appendix A - Individual Clinical Observations (cont.)

Group Sex	Animal Number	Clinical Sign	Site	Day numbers relative to Start Date														
				1	1	1	1	2	2	2	2	2	2	2	2			
3m	4084	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	4085	Excessive Hair Loss Limbs No Abnormalities Detected	Forelimb Right	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	4086	No Abnormalities Detected	Eye Left	R
	4087	Injury Apparent Mechanical No Abnormalities Detected	Forepaw Right	B	B
	4088	Injury Apparent Mechanical No Abnormalities Detected	Forepaw Right	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

X = Present
D = Dark Yellow
C = Clear
B = Broken/Missing Toe Nail
R = Red
Y = Yellow

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Appendix A - Individual Clinical Observations (cont.)

Group Sex	Animal Number	Clinical Sign	Site	Day numbers relative to Start Date														
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	
5f	4111	No Abnormalities Detected	Eye Left	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
		Soiling	Eye Bilateral
		Soiling	
4112	No Abnormalities Detected	Soiling	Eye Right	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
		Soiling	Eye Bilateral
		Soiling	
4113	No Abnormalities Detected	Soiling	Eye Right	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
		Soiling	
		Soiling	

X = Present
D = Dark Yellow
C = Clear
B = Broken/Missing Toe Nail
R = Red
Y = Yellow

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Appendix B – Individual Body Weights (grams)

Group Sex	Animal Number	Day numbers relative to Start Date					
		1	8	15	22	28	
1m	4074	238.2	250.7	262.6	277.2	286.5	
	4075	248.0	253.5	258.2	265.1	274.1	
	4076	244.2	250.9	261.5	273.7	277.8	
	4077	251.2	258.9	270.3	278.6	288.3	
	4078	241.9	245.2	254.8	264.7	275.9	
	Mean	244.70	251.84	261.48	271.86	280.52	
	S.D.	5.09	4.97	5.80	6.60	6.45	
	N	5	5	5	5	5	

Nominal Dose: Group 1 - 0 mg HMDS/kg
 Group 4 - 1000 mg HMDS/kg

Group 2 - 10 mg HMDS/kg
 Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Group 3 - 100 mg HMDS/kg
 Group 3 - 100 mg HMDS/kg

Appendix B – Individual Body Weights (grams) (cont.)

Group	Animal Sex	Day numbers relative to Start Date						
		1	8	15	22	28		
2m	4079	241.3	248.9	258.6	267.7	272.5		
	4080	234.6	235.1	243.5	254.9	260.7		
	4081	246.2	249.9	255.9	267.7	277.2		
	4082	244.3	252.0	259.2	266.4	270.9		
	4083	251.7	260.3	267.1	283.4	290.0		
	Mean	243.62	249.24	256.86	268.02	274.26		
	S.D.	6.31	9.09	8.56	10.14	10.66		
	N	5	5	5	5	5		

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, 14C HMDS on day 28

Appendix B -- Individual Body Weights (grams) (cont.)

Group Sex	Animal Number	Day numbers relative to Start Date					
		1	8	15	22	28	
3m	4084	236.3	247.6	253.9	261.8	266.5	
	4085	243.1	251.8	259.3	269.2	274.2	
	4086	245.4	248.7	256.8	268.2	273.0	
	4087	248.1	249.0	254.1	264.4	271.9	
	4088	255.5	263.8	273.4	281.5	289.7	
	Mean	245.68	252.18	259.50	269.02	275.06	
	S.D.	7.02	6.68	8.08	7.58	8.70	
	N	5	5	5	5	5	

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg

Group 2 - 10 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Group 3 - 100 mg HMDS/kg

Appendix B – Individual Body Weights (grams) (cont.)

Group Sex	Animal Number	Day numbers relative to Start Date						
		1	8	15	22	28		
4m	4090	253.0	249.9	250.1	257.1	260.9		
	4091	238.4	241.1	244.4	251.8	254.5		
	4092	251.0	246.3	250.6	258.6	262.7		
	4093	242.3	243.7	250.9	262.0	271.0		
	4099	240.5	237.3					
	Mean	245.04	243.66	249.00	257.38	262.28		
	S.D.	6.54	4.82	3.08	4.24	6.80		
	N	5	5	4	4	4		

Nominal Dose: Group 1 - 0 mg HMDS/kg
 Group 2 - 10 mg HMDS/kg
 Group 3 - 100 mg HMDS/kg
 Group 4 - 1000 mg HMDS/kg
 Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Appendix B – Individual Body Weights (grams) (cont.)

Group Sex	Animal Number	Day numbers relative to Start Date						
		1	8	15	22	28		
5m	4089	245.6	249.5	251.3	255.9	262.7		
	4094	242.2	239.9	246.2	250.5	256.0		
	4095	239.3	238.5	248.1	246.7	253.0		
	4096	232.8	229.7	232.7	240.4	243.6		
	4097	242.7	239.0	248.4	253.8	256.1		
	4098	248.0	244.7	253.2	260.8	262.5		
	4100	241.7	247.1	247.8	252.2	256.9		
	4101	256.5	257.2	262.1	271.0	279.8		
	4102	233.2	229.4	236.3	244.7	250.1		
	4103	243.6	240.6	245.0	246.2	252.6		
	Mean	242.56	241.56	247.11	252.22	257.33		
	S.D.	6.90	8.54	8.25	8.87	9.69		
	N	10	10	10	10	10		

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg

Group 2 - 10 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Group 3 - 100 mg HMDS/kg

Appendix B – Individual Body Weights (grams) (cont.)

Group Sex	Animal Number	Day numbers relative to Start Date							
		1	8	15	22	28			
5f	4104	163.0	166.7	170.9	176.5	180.5			
	4105	164.8	165.1	169.2	176.4	179.1			
	4106	160.2	159.5	164.5	166.9	167.3			
	4107	162.7	160.8	165.2	166.3	167.8			
	4108	166.7	169.6	171.0	174.9	176.5			
	4109	160.9	163.5	161.2	166.0	164.7			
	4110	160.1	159.6	159.2	161.3	162.5			
	4111	160.9	161.8	164.7	170.1	169.7			
	4112	165.5	168.6	172.7	173.6	174.3			
	4113	161.9	160.5	165.6	167.9	171.2			
	Mean	162.67	163.57	166.42	169.99	171.36			
	S.D.	2.32	3.75	4.43	5.16	6.09			
	N	10	10	10	10	10			

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Appendix C – Individual Body Weight Gain (grams)

Group Sex	Animal Number	Day numbers relative to Start Date			
		From: To:	1 8	8 15	15 22
1m	4074	12.5	11.9	14.6	9.3
	4075	5.5	4.7	6.9	9.0
	4076	6.7	10.6	12.2	4.1
	4077	7.7	11.4	8.3	9.7
	4078	3.3	9.6	9.9	11.2
		-----	-----	-----	-----
	Mean	7.14	9.64	10.38	8.66
	S.D.	3.42	2.90	3.07	2.69
	N	5	5	5	5
		-----	-----	-----	-----

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Appendix C – Individual Body Weight Gain (grams) (cont.)

Day numbers relative to Start Date

Group Sex	Animal Number	From: To:	1 8	8 15	15 22	22 28
2m	4079		7.6	9.7	9.1	4.8
	4080		0.5	8.4	11.4	5.8
	4081		3.7	6.0	11.8	9.5
	4082		7.7	7.2	7.2	4.5
	4083		8.6	6.8	16.3	6.6
		Mean	5.62	7.62	11.16	6.24
		S.D.	3.43	1.45	3.42	2.00
		N	5	5	5	5

Nominal Dose: Group 1 - 0 mg HMDS/kg Group 2 - 10 mg HMDS/kg Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Appendix C – Individual Body Weight Gain (grams) (cont.)

Day numbers relative to Start Date

Group Sex	Animal Number	From: To:	1 8	8 15	15 22	22 28
3m	4084		11.3	6.3	7.9	4.7
	4085		8.7	7.5	9.9	5.0
	4086		3.3	8.1	11.4	4.8
	4087		0.9	5.1	10.3	7.5
	4088		8.3	9.6	8.1	8.2
			-----	-----	-----	-----
	Mean		6.50	7.32	9.52	6.04
	S.D.		4.26	1.72	1.49	1.67
	N		5	5	5	5
			-----	-----	-----	-----

Nominal Dose: Group 1 - 0 mg HMDS/kg
 Group 2 - 10 mg HMDS/kg
 Group 3 - 100 mg HMDS/kg
 Group 4 - 1000 mg HMDS/kg
 Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Appendix C – Individual Body Weight Gain (grams) (cont.)

Group Sex	Animal Number	Day numbers relative to Start Date					
		From: To:	1 8	8 15	15 22	22 28	
4m	4090		-3.1	0.2	7.0	3.8	
	4091		2.7	3.3	7.4	2.7	
	4092		-4.7	4.3	8.0	4.1	
	4093		1.4	7.2	11.1	9.0	
	4099		-3.2	.	.	.	
	Mean		-1.38	3.75	8.38	4.90	
	S.D.		3.23	2.89	1.86	2.80	
	N		5	4	4	4	

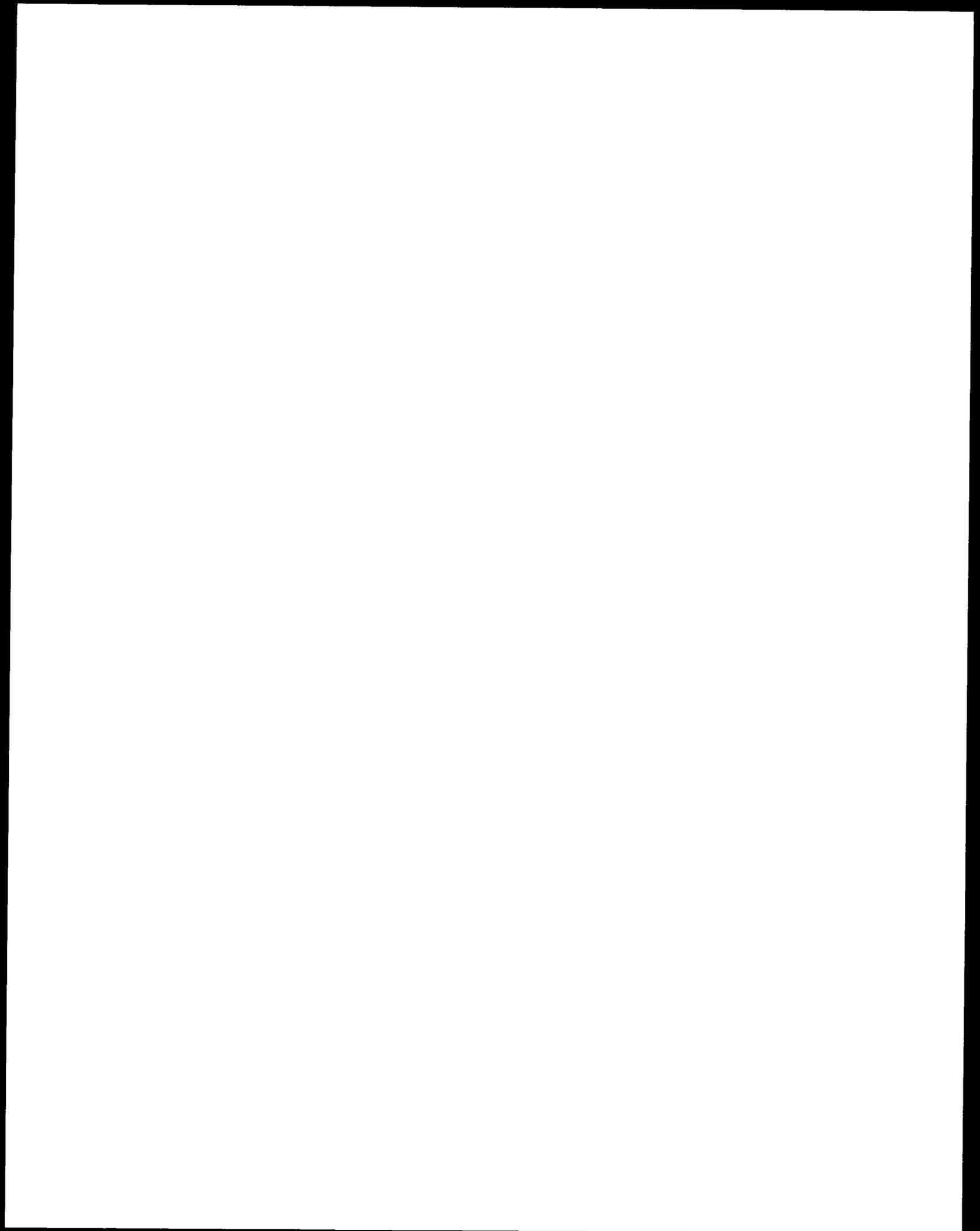
Nominal Dose: Group 1 - 0 mg HMDS/kg
 Group 2 - 10 mg HMDS/kg
 Group 3 - 100 mg HMDS/kg
 Group 4 - 1000 mg HMDS/kg
 Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

Appendix C -- Individual Body Weight Gain (grams) (cont.)

Day numbers relative to Start Date

Group Sex	Animal Number	From:		1		8		15		22		28	
		To:	8	15	22	28	15	22	28	15	22	28	
5m	4089		3.9	1.8	4.6	6.8							
	4094		-2.3	6.3	4.3	5.5							
	4095		-0.8	9.6	-1.4	6.3							
	4096		-3.1	3.0	7.7	3.2							
	4097		-3.7	9.4	5.4	2.3							
	4098		-3.3	8.5	7.6	1.7							
	4100		5.4	0.7	4.4	4.7							
	4101		0.7	4.9	8.9	8.8							
	4102		-3.8	6.9	8.4	5.4							
	4103		-3.0	4.4	1.2	6.4							
	Mean		-1.00	5.55	5.11	5.11							
	S.D.		3.31	3.13	3.29	2.19							
	N		10	10	10	10							

Nominal Dose: Group 1 - 0 mg HMDS/kg
Group 2 - 10 mg HMDS/kg
Group 3 - 100 mg HMDS/kg
Group 4 - 1000 mg HMDS/kg
Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28



Appendix C - Individual Body Weight Gain (grams) (cont.)

Day numbers relative to Start Date

Group Sex	Animal Number	From: To:	1 8	8 15	15 22	22 28	
5f	4104		3.7	4.2	5.6	4.0	
	4105		0.3	4.1	7.2	2.7	
	4106		-0.7	5.0	2.4	0.4	
	4107		-1.9	4.4	1.1	1.5	
	4108		2.9	1.4	3.9	1.6	
	4109		2.6	-2.3	4.8	-1.3	
	4110		-0.5	-0.4	2.1	1.2	
	4111		0.9	2.9	5.4	-0.4	
	4112		3.1	4.1	0.9	0.7	
	4113		-1.4	5.1	2.3	3.3	
				-----	-----	-----	-----
		Mean		0.90	2.85	3.57	1.37
		S.D.		2.04	2.50	2.13	1.64
	N		10	10	10	10	
			-----	-----	-----	-----	

Nominal Dose: Group 1 - 0 mg HMDS/kg
 Group 2 - 10 mg HMDS/kg
 Group 3 - 100 mg HMDS/kg
 Group 4 - 1000 mg HMDS/kg
 Group 5 - 1000 mg HMDS/kg, ¹⁴C HMDS on day 28

**DOW CORNING CORPORATION
HEALTH & ENVIRONMENTAL SCIENCES**

CONTRIBUTING SCIENTIST REPORT

Study Title: NON-REGULATED STUDY: Hexamethyldisiloxane (HMDS): Determination of the Reversible Binding of HMDS/Metabolites to Alpha 2u-Globulin in Male Fischer 344 Rats Following Oral Gavage Administration

Study Number: HES Study Number: 10461-101

Test Article: Hexamethyldisiloxane (HMDS)

Contributing Scientist: James W. Crissman, D.V.M., Ph.D.,
Diplomate, American College of Veterinary Pathologists
Crissman Toxicologic Pathology, LLC
Midland, MI

Study Director: Jeanne Y. Domoradzki, Ph.D., D.A.B.T.
Study Leader, Toxicology
Health and Environmental Sciences

Sponsor: Dow Corning Corporation
2200 W. Salzburg Rd, Auburn, MI 48611

Testing Facility: Dow Corning Corporation
Health and Environmental Sciences
2200 West Salzburg Road
Auburn, Michigan 48611

TABLE OF CONTENTS

Page Number:

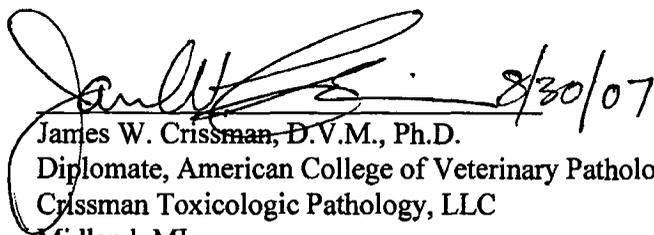
TITLE PAGE	D1
TABLE OF CONTENTS	D2
APPROVAL SIGNATURES	D3
METHODS	D4
RESULTS AND DISCUSSION	D4
Intergroup Comparison of Gross Pathology Observations (Table 1).....	D6
Intergroup Comparison of Histopathology Observations (Table 2).....	D17
Histopathology Observations - Animal Cross Reference (Table 3).....	D18
Individual Animal Data (Table 4).....	D19

APPROVAL SIGNATURES

Report Title: Morphologic Kidney Pathology Findings for
Hexamethyldisiloxane (HMDS): Determination of the
Reversible Binding of HMDS/Metabolites to Alpha 2u-
Globulin in Male Fischer 344 Rats Following Oral Gavage
Administration

Submitted by: Dow Corning Corporation
Health and Environmental Sciences (HES)
2200 W. Salzburg Rd.
Auburn, Michigan 48611

Approved by:


James W. Crissman, D.V.M., Ph.D.
Diplomate, American College of Veterinary Pathologists
Crissman Toxicologic Pathology, LLC
Midland, MI


Paul A. Jean, Ph.D.
Team Leader, Toxicology
Health and Environmental Sciences
Dow Corning Corporation

METHODS

For the histopathology segment of this study, four or five (see table) male F-344 rats were orally gavaged with HMDS in corn oil at 0, 10, 100, or 1000 mg/kg/day for 28 consecutive days. Animals were euthanized approximately 2 hours after the final administration of the test article. Their kidneys were collected and fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin, sectioned at approximately 5 microns, stained with hematoxylin and eosin (H&E), and examined by light microscopy.

Group Number	Number of Animals	Treatment	mg HMDS/kg BW
1	4	Corn Oil	0
2	5	HMDS	10
3	5	HMDS	100
4	4	HMDS	1000

RESULTS AND DISCUSSION

Gross Pathology

The incidence of gross pathology findings is summarized in Table 1; individual data may be found in Table 4. There were no gross pathological changes attributable to the test material.

Microscopic Pathology

The incidence of microscopic pathology findings is summarized in Table 2; individual data may be found in Tables 3 and 4.

At 10 mg/kg/day there was a possible small increase in minimal focal or multifocal tubular basophilia (controls, 2/4; 10 mg/kg/day, 5/5). This is a common spontaneous finding in male rats of this age and strain, but it also indicates a reparative response for replacement of renal cortical epithelial cells. The cellular basophilia is a reflection of fairly recent cell division. The amount and histomorphology of protein droplet accumulation in the cortex did not appear different from controls.

At 100 mg/kg/day the amount and degree of tubular basophilia was similar to that seen at 10 mg/kg/day. Alpha 2u-globulin (A2uG) nephropathy was graded as minimal in all of these rats. The only indication of the effect visible in these sections was the occasional angular cytoplasmic inclusion in the P2 proximal tubule segments where the A2uG protein is normally visible and where it is resorbed. In normal male rat kidneys there are many eosinophilic droplets in radial segments at regular intervals around the cortex. In these segments, the lysosomes containing the resorbed A2uG protein are normally visible as rounded eosinophilic cytoplasmic inclusions. The smallest recognizable change with minimal A2uG nephropathy is crystallization of the material in these inclusions, giving them an angular appearance. This is a very subtle change.

At 1000 mg/kg/day there was moderate multifocal tubular basophilia in all sections, indicating a substantial reparative process. Alpha 2u globulin nephropathy was graded as moderate in all sections. In addition to the increase in multifocal tubular basophilia, the nephropathy was characterized by an increase in the portion of the cortex with tubular luminal protein droplets and epithelial cytoplasmic inclusions. Most of the latter had angular crystalline shapes. Also, there were one to several large eosinophilic granular casts at the cortico-medullary junction in all sections. These represent accumulated dead cortical tubule epithelial cells and are typical of this nephropathy. Scattered focal mineralization of the outer medulla was also observed in some cases.

Grading Scheme:

Grade-1, minimal. Tissue changes are minimally distinguishable from normal.

Grade-2, mild. Tissues changes are readily distinguishable, but not expected to significantly affect organ function, clinical pathology parameters, or clinical signs.

Grade-3, moderate. Tissue changes are easily appreciated and may have significant effects on organ function, clinical pathology parameters, or clinical signs.

Grade-4, severe. Tissue changes are extensive and will markedly affect organ function, perhaps to the extent of organ failure. Severe lesions in vital organs may cause or contribute to the death of the animal.

REFERENCES

Khan, K. N. M. and Alden, C. L. (2002). Kidney. In: Handbook of Toxicologic Pathology, 2nd Edition, Vol. 2. Editors: W. M. Haschek, C. G. Rousseaux, M. A. Wallig. Academic Press, San Diego. pp. 294-299.

Table 1. Intergroup Comparison of Gross Pathology Observations

Removal Reasons: All of those SELECTED	MALES				
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg HMDS	1000 mg/kg HMDS	10000 mg/kg HMDS
ADRENAL GLANDS;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
AORTA;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
BRAIN;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
CAVITY, ABDOMINAL;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
CAVITY, CRANIAL;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
CAVITY, ORAL;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
CAVITY, THORACIC;					
Submitted.....	(4)	(5)	(5)	(5)	(5)
No Visible Lesions.....	4	5	5	5	4
Fibrin.....	0	0	0	0	1
Fluid; cloudy.....	0	0	0	0	1
EARS;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
EPIDIDYMIDES;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4

Table 1. Intergroup Comparison of Gross Pathology Observations (Con't)

Removal Reasons: All of those SELECTED	0 mg/kg				10 mg/kg				100 mg/kg				1000 mg/kg			
	HMDS	HMDS	HMDS	HMDS	HMDS	HMDS	HMDS	HMDS	HMDS	HMDS	HMDS	HMDS	HMDS	HMDS	HMDS	HMDS
ESOPHAGUS;																
Submitted.....	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5
No Visible Lesions.....	(4)	(4)	(4)	(4)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
EYES;																
Submitted.....	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5
No Visible Lesions.....	(4)	(4)	(4)	(4)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
ADIPOSE TISSUE;																
Submitted.....	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5
No Visible Lesions.....	(4)	(4)	(4)	(4)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
HEART;																
Submitted.....	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5
No Visible Lesions.....	(4)	(4)	(4)	(4)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
INTESTINE, CECUM;																
Submitted.....	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5
No Visible Lesions.....	(4)	(4)	(4)	(4)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
INTESTINE, COLON;																
Submitted.....	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5
No Visible Lesions.....	(4)	(4)	(4)	(4)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
INTESTINE, DUODENUM;																
Submitted.....	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5
No Visible Lesions.....	(4)	(4)	(4)	(4)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
INTESTINE, ILEUM;																
Submitted.....	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5
No Visible Lesions.....	(4)	(4)	(4)	(4)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
INTESTINE, JEJUNUM;																
Submitted.....	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5
No Visible Lesions.....	(4)	(4)	(4)	(4)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
INTESTINE, RECTUM;																
Submitted.....	4	4	4	4	5	5	5	5	5	5	5	5	5	5	5	5
No Visible Lesions.....	(4)	(4)	(4)	(4)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)

Table 1. Intergroup Comparison of Gross Pathology Observations (Con't)

Removal Reasons: All of those SELECTED	MALES			
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg HMDS	1000 mg/kg HMDS
INTESTINE, RECTUM; (continued)				
No Visible Lesions.....	4	5	5	4
KIDNEYS;				
Submitted.....	(4)	(5)	(5)	(5)
No Visible Lesions.....	4	5	5	5
LARYNX;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
LIVER;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
LUNGS;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
LYMPH NODE, MEDIASTINAL;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
LYMPH NODE, MESENTERIC;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
SKELETAL MUSCLE;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
NERVE;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
NOSE;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4

Table 1. Intergroup Comparison of Gross Pathology Observations (Con't)

Removal Reasons: All of those SELECTED	MALES				
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg HMDS	1000 mg/kg HMDS	1000 mg/kg HMDS
	4	5	5	5	4
Number of Animals on Study :	(4)	(5)	(5)	(5)	(4)
Number of Animals Completed:	(4)	(5)	(5)	(5)	(4)
PANCREAS;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
PITUITARY GLAND;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
PROSTATE GLAND;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
SEMINAL VESICLES;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
SKIN;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
SPLEEN;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4
STOMACH;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	3
Erosion; glandular; multiple.....	0	0	0	0	1
TESTES;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	3
Decreased Size; unilateral.....	0	0	0	0	1
THYMUS;					
Submitted.....	(4)	(5)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	5	4

Table 1. Intergroup Comparison of Gross Pathology Observations (Con't)

Removal Reasons: All of those SELECTED	----- MALES -----			
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg HMDS	1000 mg/kg HMDS
Number of Animals on Study :	4	5	5	4
Number of Animals Completed:	(4)	(5)	(5)	(4)
THYROID GLANDS;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
TONGUE;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
TEETH;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
TRACHEA;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
URINARY BLADDER;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4
WHOLE BODY;				
Submitted.....	(4)	(5)	(5)	(4)
No Visible Lesions.....	4	5	5	4

Table 2. Summary of Microscopic Pathology Findings

Observations: Neo-Plastic and Non Neo-Plastic Removal Reasons: All of those SELECTED	----- MALES -----			
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg g HMDS	1000 mg/kg k HMDS
KIDNEYS;				
Examined.....	(4)	(5)	(5)	(4)
Within Normal Limits.....	2	0	0	0
Inflammation; chronic; focal.....	(0)	(0)	(0)	(1)
minimal.....	0	0	0	1
Inflammation; chronic; multifocal.....	(0)	(1)	(0)	(2)
minimal.....	0	0	0	2
mild.....	0	1	0	0
Basophilic; tubular; focal.....	(2)	(3)	(3)	(0)
minimal.....	2	3	3	0
Basophilic; tubular; multifocal.....	(0)	(2)	(2)	(4)
minimal.....	0	2	2	0
moderate.....	0	0	0	4
Nephropathy; alpha 2u globulin.....	(0)	(0)	(5)	(4)
minimal.....	0	0	5	0
moderate.....	0	0	0	4

Table 3. Individual Microscopic Pathology Findings

Removal Reason: All of those selected	MALES			
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg HMDS	1000 mg/kg HMDS
KIDNEYS;				
Examined	(4)	(5)	(5)	(4)
Within Normal Limits	4077 4078			
Inflammation; chronic; focal; minimal		4079		4091
Inflammation; chronic; multifocal; minimal			4080 4081 4082	4092 4093
mild				
Basophililia; tubular; focal; minimal	4075 4076		4084 4086 4088	
Basophililia; tubular; multifocal; minimal		4079 4083	4085 4087	4090 4091 4092 4093
moderate				
Nephropathy; alpha 2u globulin; minimal			4084 4085 4086 4087 4088	
moderate				4090 4091 4092 4093

Table 4. Individual Gross Pathology Findings

Removal Reason: All of those selected

	----- MALES -----			
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg g HMDS	1000 mg/kg kg HMDS
ADRENAL GLANDS;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
AORTA;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
BRAIN;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
CAVITY, ABDOMINAL;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
CAVITY, CRANIAL;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	

Table 4. Individual Gross Pathology Findings (Con't)

	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg g HMDS	1000 mg/kg kg HMDS
CAVITY, ORAL;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075 4076 4077 4078	4079 4080 4081 4082 4083	4084 4085 4086 4087 4088	4090 4091 4092 4093
CAVITY, THORACIC;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075 4076 4077 4078	4079 4080 4081 4082 4083	4084 4085 4086 4087 4088	4090 4091 4092 4093
EARS;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075 4076 4077 4078	4079 4080 4081 4082 4083	4084 4085 4086 4087 4088	4090 4091 4092 4093
EPIDIDYMIDES;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075 4076 4077 4078	4079 4080 4081 4082 4083	4084 4085 4086 4087 4088	4090 4091 4092 4093
ESOPHAGUS;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075 4076 4077 4078	4079 4080 4081 4082 4083	4084 4085 4086 4087 4088	4090 4091 4092 4093

Table 4. Individual Gross Pathology Findings (Con't)

Removal Reason: All of those selected	MALES			
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg HMDS	1000 mg/kg HMDS
EYES;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
ADIPOSE TISSUE;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
HEART;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
INTESTINE, CECUM;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
INTESTINE, COLON;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	

Table 4. Individual Gross Pathology Findings (Cont)

Removal Reason: All of those selected	MALES			
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg HMDS	1000 mg/kg HMDS
INTESTINE, DUODENUM;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
INTESTINE, ILEUM;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
INTESTINE, JEJUNUM;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
INTESTINE, RECTUM;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
KIDNEYS;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	

Table 4. Individual Gross Pathology Findings (Con't)

Removal Reason: All of those selected	----- MALES -----					
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg g HMDS	1000 mg/kg kg HMDS	(4)	(5)
LARYNX;						
Submitted	(4)	(5)	(5)	(4)		
No Visible Lesions	4075	4079	4084	4090		
	4076	4080	4085	4091		
	4077	4081	4086	4092		
	4078	4082	4087	4093		
		4083	4088			
LIVER;						
Submitted	(4)	(5)	(5)	(4)		
No Visible Lesions	4075	4079	4084	4090		
	4076	4080	4085	4091		
	4077	4081	4086	4092		
	4078	4082	4087	4093		
		4083	4088			
LUNGS;						
Submitted	(4)	(5)	(5)	(4)		
No Visible Lesions	4075	4079	4084	4090		
	4076	4080	4085	4091		
	4077	4081	4086	4092		
	4078	4082	4087	4093		
		4083	4088			
SKELETAL MUSCLE;						
Submitted	(4)	(5)	(5)	(4)		
No Visible Lesions	4075	4079	4084	4090		
	4076	4080	4085	4091		
	4077	4081	4086	4092		
	4078	4082	4087	4093		
		4083	4088			
NERVE;						
Submitted	(4)	(5)	(5)	(4)		
No Visible Lesions	4075	4079	4084	4090		
	4076	4080	4085	4091		
	4077	4081	4086	4092		
	4078	4082	4087	4093		
		4083	4088			

Table 4. Individual Gross Pathology Findings (Con't)

Removal Reason: All of those selected	MALES			
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg HMDS	1000 mg/kg HMDS
NOSE;				
Submitted	(4) 4075	(5) 4079	(5) 4084	(4) 4090
No Visible Lesions	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
PANCREAS;				
Submitted	(4) 4075	(5) 4079	(5) 4084	(4) 4090
No Visible Lesions	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
PITUITARY GLAND;				
Submitted	(4) 4075	(5) 4079	(5) 4084	(4) 4090
No Visible Lesions	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
PROSTATE GLAND;				
Submitted	(4) 4075	(5) 4079	(5) 4084	(4) 4090
No Visible Lesions	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
SEMINAL VESICLES;				
Submitted	(4) 4075	(5) 4079	(5) 4084	(4) 4090
No Visible Lesions	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	

Table 4. Individual Gross Pathology Findings (Cont)

Removal Reason: All of those selected	MALES					
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg g HMDS	1000 mg/kg kg HMDS	1000 mg/kg kg HMDS	
SKIN;						
Submitted	(4)	(5)	(5)	(4)		
No Visible Lesions	4075	4079	4084	4090		
	4076	4080	4085	4091		
	4077	4081	4086	4092		
	4078	4082	4087	4093		
		4083	4088			
SPLEEN;						
Submitted	(4)	(5)	(5)	(4)		
No Visible Lesions	4075	4079	4084	4090		
	4076	4080	4085	4091		
	4077	4081	4086	4092		
	4078	4082	4087	4093		
		4083	4088			
STOMACH;						
Submitted	(4)	(5)	(5)	(4)		
No Visible Lesions	4075	4079	4084	4090		
	4076	4080	4085	4092		
	4077	4081	4086	4093		
	4078	4082	4087			
		4083	4088			
						4091
glandular; Erosion; multiple						
TESTES;						
Submitted	(4)	(5)	(5)	(4)		
No Visible Lesions	4075	4079	4084	4091		
	4076	4080	4085	4092		
	4077	4081	4086	4093		
	4078	4082	4087			
		4083	4088			
Decreased Size; unilateral						4090
THYMUS;						
Submitted	(4)	(5)	(5)	(4)		
No Visible Lesions	4075	4079	4084	4090		
	4076	4080	4085	4091		

Table 4. Individual Gross Pathology Findings (Con't)

Removal Reason: All of those selected	----- MALES -----			
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg 9 HMDS	1000 mg/kg 1000 mg/ kg HMDS
THYMUS; (continued)				
No Visible Lesions (continued)	4077 4078	4081 4082 4083	4086 4087 4088	4092 4093
THYROID GLANDS;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
TONGUE;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
TEETH;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
TRACHEA;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090
	4076	4080	4085	4091
	4077	4081	4086	4092
	4078	4082	4087	4093
		4083	4088	
URINARY BLADDER;				
Submitted	(4)	(5)	(5)	(4)
No Visible Lesions	4075	4079	4084	4090

Table 4. Individual Gross Pathology Findings (Con't)

Removal Reason: All of those selected	----- MALES -----					
	0 mg/kg HMDS	10 mg/kg HMDS	100 mg/kg g HMDS	1000 mg/kg kg HMDS	1000 mg/kg kg HMDS	
URINARY BLADDER; (continued)						
No Visible Lesions (continued)	4076	4080	4085	4091		
	4077	4081	4086	4092		
	4078	4082	4087	4093		
		4083	4088			
WHOLE BODY;						
Submitted	(4)	(5)	(5)	(4)		
No Visible Lesions	4075	4079	4084	4090		
	4076	4080	4085	4091		
	4077	4081	4086	4092		
	4078	4082	4087	4093		

Appendix E

DOW CORNING CORPORATION HEALTH & ENVIRONMENTAL SCIENCES

CONTRIBUTING SCIENTIST REPORT

Study Title: **NON-REGULATED STUDY:** Study of Hexamethyldisiloxane (HMDS): Determination of the Reversible Binding of HMDS/Metabolites to Alpha 2u-Globulin in Male Fischer 344 Rats Following Oral Gavage Administration

Study Number: HES Study Number: 10461-101

Test Article: ¹⁴C- Hexamethyldisiloxane

Contributing Scientists: Jacob M. Sushynski, B.S.
Senior Analytical Chemist

Debra A. McNett, B.S.
Bioanalytical Supervisor

Study Leader: Jeanne Y. Domoradzki, Ph.D., D.A.B.T.
Toxicology Specialist

Sponsor: Dow Corning Corporation
2200 W. Salzburg Road
Auburn, MI 48611

Testing Facility: Dow Corning Corporation
Health and Environmental Sciences
2200 West Salzburg Road
Auburn, Michigan 48611

TABLE OF CONTENTS

TABLE OF CONTENTS..... E2
APPROVAL SIGNATURES..... E3
OBJECTIVE E4
MATERIALS AND METHODS..... E4
 1. Validation..... E4
 2. Study Sample Collections, Processing and Analysis..... E4
 3. Parameters Evaluated..... E5
 4. Statistical Analysis..... E5
RESULTS AND DISCUSSION E5
 1. Study Samples..... E5
 2. Results and Discussion..... E6
SUMMARY E6
ARCHIVE..... E7
REFERENCES E7

LIST OF TABLES

Table 1 - Concentration of Parent and Radioactivity and Percentage of
 Parent/Radioactivity at 2 h Post-Dosing..... E8
Table 2 - Radioactivity Burden at 2 h Post-Dosing..... E9

LIST OF FIGURES

Figure 1 - Parent HMDS and Metabolite(s) in Rat Tissues; Average of Group 5 Males and
 Females, 2 h Post-DosingE10

LIST OF ATTACHMENTS

ATTACHMENT A - Procedure for Determination of HMDS in Biological Matrices
 (Blood and Kidney)..... E11
ATTACHMENT B - Individual Sample Values E20

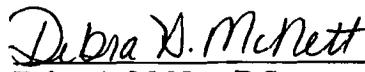
APPROVAL SIGNATURES

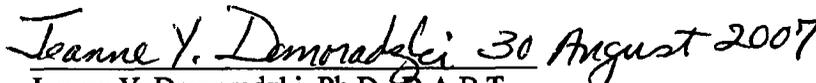
Report Title: **NON-REGULATED STUDY: Study of Hexamethyldisiloxane (HMDS): Determination of the Reversible Binding of HMDS/Metabolites to Alpha 2u-Globulin in Male Fischer 344 Rats Following Oral Gavage Administration**

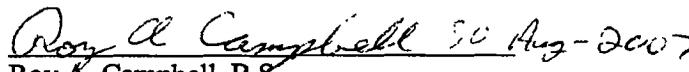
Submitted by: Dow Corning Corporation
Health and Environmental Sciences (HES)
2200 W. Salzburg Rd.
Auburn, Michigan 48611

Approved by:


3 Aug 2007
Jacob M. Sushynski, B.S.
Senior Analytical Chemist


30-Aug-2007
Debra A. McNett, B.S.
Bioanalytical Supervisor


30 August 2007
Jeanne Y. Domoradzki, Ph.D., D.A.B.T.
Toxicology Specialist
Study Leader


30 Aug-2007
Roy A. Campbell, B.S.
Health and Environmental Sciences
HES Group Manager - Chemistry and
Environmental

OBJECTIVE

The objective of this study was to evaluate alpha 2u-nephropathy induction and the reversible binding of alpha 2u-globulin to HMDS and/or metabolites in male rat kidneys following oral gavage administration of HMDS in a corn oil vehicle to male and female (control) animals for 28 days over a target dose level range of 10 to 1000 mg/kg body weight. The concentrations of parent HMDS and total radioactivity in rat were determined in blood and kidney from the 1000 mg/kg dose group, Group 5.

MATERIALS AND METHODS

1. Validation

A method was validated for the extraction and quantitative analysis of blood and kidney for the quantitation of HMDS. The validated method was based upon the principle of calibrating a GC/MS using spiked solvent standards in tetrahydrofuran (THF), and then running extracts of spiked and blank control blood and kidney samples against this calibration curve. Extractions of the spikes and blanks were done with THF containing a stable isotope internal standard ¹³C-Hexamethyldisiloxane (¹³C-HMDS). In this manner, an assessment of precision, accuracy, linearity, specificity/selectivity, limit of detection (LOD), and limit of quantitation (LOQ) were derived. These aspects of the method that were assessed can be collectively referred to as figures of merit. The method was similar to a method that had been previously validated for the analysis of HMDS in blood and tissues. (HES study 9829).

2. Study Sample Collections, Processing and Analysis

The test substance (unlabeled) in corn oil vehicle at the targeted dose was introduced by gavage directly into the stomachs of the study animals for 27 consecutive days. On day 28, the test substance (¹⁴C-labeled, specific activity xx mCi/g) in corn oil vehicle at the targeted dose was introduced by gavage directly into the stomachs of the study animals. Terminal blood was collected via cardiac puncture 2-hours following the day 28 dose administration, and kidneys were also collected. One kidney was used from each animal for the determination of parent and radioactivity.

Parent Analysis

Blood samples were extracted twice with THF. Extraction of the kidney samples was accomplished with a single THF extraction. The THF extractions incorporated the internal standard ¹³C-Hexamethyldisiloxane (¹³C-HMDS) for the analysis. Parent HMDS was quantitated in the THF extracts of blood and kidney against THF solvent standards using gas

chromatography-mass spectrometry GC/MS, according to the validated method "Procedure for Determination of HMDS in Biological Matrices (Blood and Kidney)". This procedure is included in **Attachment A**.

Radioactivity Analysis

Radioactivity was quantitated in the THF extracts of blood and kidney by direct analysis using a liquid scintillation analyzer. In addition, the remaining pellet after THF extraction was solubilized using 35% tetraethyl ammonium hydroxide (TEAH). Aliquots of the solubilized pellet were processed for liquid scintillation analysis to determine the amount of radioactivity remaining after extraction.

3. Parameters Evaluated

Parent HMDS concentrations were measured in the blood and kidney. The concentration of parent HMDS in rat tissues was reported as μg HMDS/gram sample. The calculations used to convert from peak areas generated from the GC/MS to μg HMDS/gram sample can be found in the procedure in **Attachment A**. Total radioactivity concentrations were measured in blood and kidney. The concentrations were reported as μg ^{14}C -HMDS equivalents per gram sample. The calculation of ^{14}C -HMDS equivalents was based upon the specific activity of the dose solution administered.

4. Statistical Analysis

Numerical data obtained during the conduct of the study was processed using Provantis and Microsoft ExcelTM Version 11 and subjected to calculation of the parameters indicated above. This included group mean values and standard errors of the means, where appropriate. Rounding differences in tables may occur due to the calculation of numbers using more significant figures than are shown.

RESULTS AND DISCUSSION

1. Study Samples

The blood and kidney samples along with a set of QC samples for each matrix were prepared on the day of processing and extracted according to the validated method. The individual results from the analysis of the samples and prepared QC spikes are presented in **Attachment B**. All of the QC samples within the analyzed range of the samples had accuracies within 20% of the prepared concentrations.

2. Results and Discussion

HMDS administered in a corn oil solution at a targeted dose of 1000 mg/kg bw was absorbed, distributed and metabolized as evidenced by observed radioactivity and parent in blood and kidney, (Tables 1-2).

Average blood and kidney samples contained approximately 30 and 50% of the total radioactivity, respectively, as parent material (Table 1) indicating that metabolism of HMDS in rat did occur. The parent analysis utilized a stable-isotope internal standard where any loss due to the extraction procedure was corrected for by proportionally effecting both analyte and internal standard equally. The radioactivity analysis is not corrected for any potential loss due to processing. A single radioactive dose was given so parent HMDS cannot be greater than the total radioactivity.

The parent HMDS and total radioactivity expressed as total burden in each tissue determined at 2-h post-administration are displayed in Table 2. A graph of the parent and total radioactivity recovered in each of the tissues (mean values of males and females) is presented in Figure 1.

The mean measured concentrations of parent and radioactivity in blood were very similar between male and female test groups. Males were found to have on average 13.5 µg/g of parent and 49.2 µg/g equivalents of total radioactivity in blood. Females were found to have on average 15.0 µg/g of parent and 48.5 µg/g equivalents of total radioactivity in blood.

The mean measured concentrations of parent and radioactivity in kidney were dissimilar between male and female test groups. Males were found to have on average 120 µg/g of parent and 232 µg/g equivalents of total radioactivity in kidney. Females were found to have on average 58.7 µg/g of parent and 121 µg/g equivalents of total radioactivity in kidney. These measured concentrations in kidney show a two-fold higher concentration of parent and total radioactivity in male kidney versus female kidney.

Even though total concentrations for parent and total radioactivity in kidney were not alike between dose groups, the ratio of parent to total radioactivity for both groups was essentially 50%.

SUMMARY

A sensitive and selective THF extraction method was validated for determining levels of HMDS in rat blood and tissues. The method was then used to determine parent HMDS and radioactivity concentrations in rat blood and kidney following a oral gavage administration of ¹⁴C-HMDS in corn oil at 1000 mg HMDS/kg bw.

ARCHIVE

All raw data and a reference substance retention sample associated with samples analysis are archived at Dow Corning Corporation, Health and Environmental Sciences, 2200 W. Salzburg Road, Auburn, MI 48611.

REFERENCES

1. DCC Study# 9829, Study of Hexamethyldisiloxane (HMDS) in the Rat Following a 14-Day Nose-Only Vapor Inhalation Exposure to HMDS Followed by an Exposure to ¹⁴C-HMDS on Day 15, Additionally Studied is a Single Nose-Only Vapor Inhalation Exposure to ¹⁴C-HMDS, DCC Report #2006-I0000-55952.

Table 1 – Concentration of Parent and Radioactivity and Percentage of Parent/Radioactivity at 2 h Post-Dosing

Sample ID	Blood			Kidney		
	Parent µg/g	Total Rad µg eq/g	Parent as % of Total	Parent µg/g	Total Rad µg eq/g	Parent as % of Total
4094	13.9	52.1	26.6%	137	266	51.6%
4095	16.1	49.0	32.7%	146	250	58.2%
4096	13.1	49.1	26.7%	127	250	50.7%
4097	16.6	50.8	32.6%	137	260	52.6%
4098	11.7	48.2	24.2%	111	220	50.4%
4089	12.5	42.2	29.5%	118	208	56.7%
4100	14.3	54.9	26.0%	123	267	45.8%
4101	6.88	44.1	15.6%	80.8	172	47.1%
4102	11.0	40.6	27.1%	93.8	165	56.9%
4103	18.9	60.7	31.2%	131	261	50.3%
Average	13.5	49.2	27.2%	120	232	52.0%

Sample ID	Blood			Kidney		
	Parent µg/g	Total Rad µg eq/g	Parent as % of Total	Parent µg/g	Total Rad µg eq/g	Parent as % of Total
4104	11.7	39.3	29.8%	39.2	85.3	45.9%
4105	14.0	46.8	30.0%	52.7	110	47.8%
4106	16.0	48.4	33.1%	64.1	127	50.5%
4107	15.1	49.5	30.6%	56.8	115	49.2%
4108	13.0	42.0	30.9%	46.0	89.2	51.6%
4109	16.2	50.6	32.1%	65.9	150	44.0%
4110	12.5	49.9	25.1%	58.4	134	43.7%
4111	17.8	52.3	34.1%	66.8	122	54.7%
4112	17.3	60.2	28.7%	70.1	154	45.4%
4113	16.4	46.1	35.6%	66.9	119	56.3%
Average	15.0	48.5	31.0%	58.7	121	48.9%

Sample ID 4094 - 4103 (including 4089) are male Fischer 344 rats.
Sample ID 4104 - 4113 are female Fischer 344 rats.

Table 2 - Radioactivity Burden at 2 h Post-Dosing

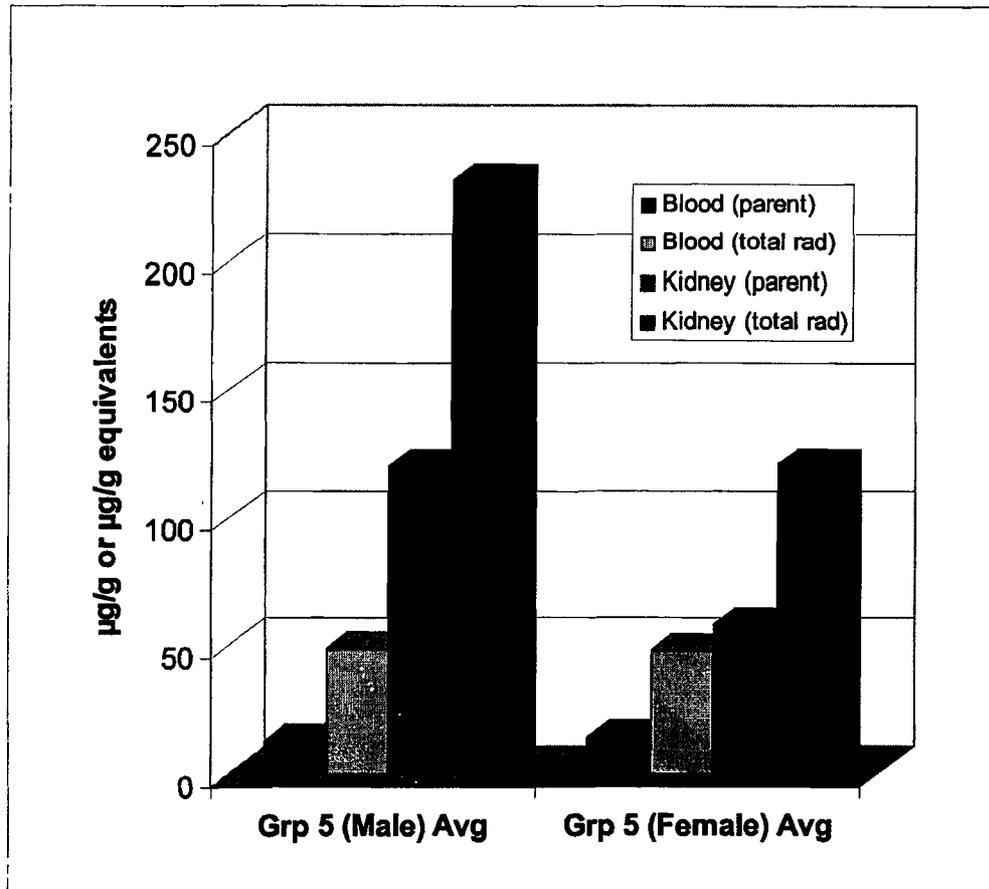
Sample ID	Blood				Kidney			
	Parent total µg	Metabolite(s) total µg	Total Rad total µg eq	% Parent	Parent total µg	Metabolite(s) total µg	Total Rad total µg eq	% Parent
4094	3.63	10.0	13.6	26.6%	110	104	214	51.6%
4095	4.01	8.25	12.3	32.7%	118	84.5	202	58.2%
4096	3.41	9.35	12.8	26.7%	96.3	93.8	190	50.7%
4097	4.55	9.39	13.9	32.6%	117	106	223	52.6%
4098	3.18	9.95	13.1	24.2%	105	103	208	50.4%
4089	3.29	7.86	11.2	29.5%	104	79.5	184	56.7%
4100	4.99	14.2	19.2	26.0%	92.9	110	203	45.8%
4101	1.82	9.87	11.7	15.6%	75.1	84.4	159	47.1%
4102	2.90	7.81	10.7	27.1%	83.1	62.8	146	56.9%
4103	5.14	11.3	16.5	31.2%	108	106	214	50.3%
Average	3.69	9.80	13.5	27.2%	101	93.4	194	52.0%

Sample ID	Blood				Kidney			
	Parent total µg	Metabolite(s) total µg	Total Rad total µg eq	% Parent	Parent total µg	Metabolite(s) total µg	Total Rad total µg eq	% Parent
4104	3.15	7.41	10.6	29.8%	22.9	27.0	49.8	45.9%
4105	3.70	8.66	12.4	30.0%	31.0	33.8	64.9	47.8%
4106	4.12	8.32	12.4	33.1%	31.2	30.6	61.8	50.5%
4107	3.98	9.03	13.0	30.6%	30.6	31.7	62.3	49.2%
4108	3.42	7.66	11.1	30.9%	26.5	24.9	51.4	51.6%
4109	4.35	9.18	13.5	32.1%	34.4	43.7	78.2	44.0%
4110	3.24	9.66	12.9	25.1%	30.7	39.5	70.2	43.7%
4111	4.90	9.48	14.4	34.1%	35.1	29.1	64.3	54.7%
4112	4.68	11.6	16.3	28.7%	36.9	44.4	81.3	45.4%
4113	4.47	8.08	12.5	35.6%	35.3	27.4	62.7	56.3%
Average	4.00	8.91	12.9	31.0%	31.5	33.2	64.7	48.9%

Sample ID 4094 - 4103 (including 4089) are male Fischer 344 rats.

Sample ID 4104 - 4113 are female Fischer 344 rats.

**Figure 1 – Parent HMDS and Metabolite(s) in Rat Tissues;
Average of Group 5 Males and Females, 2 h Post-Dosing**



ATTACHMENT A

**Procedure for Determination of HMDS in Biological Matrices
(Blood and Kidney)**

STUDY 10461



**Jacob M. Sushynski, B.S.
Senior Analytical Chemist**

5/9/07

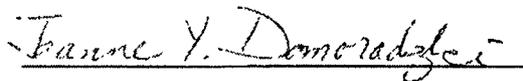
Date



**Debra A. McNett, B.S.
Bioanalytical Supervisor**

May 9, 2007

Date



**Jeanne Y. Domoradzki, Ph.D.
Study Director**

May 9, 2007

Date

**PROCEDURE FOR DETERMINATION OF HMDS IN BIOLOGICAL
MATRICES
(Blood and Kidney)**

PURPOSE

To describe a method for extraction and quantification of Hexamethyldisiloxane (HMDS) in biological matrices.

EQUIPMENT

1. Chemicals

- | | |
|---|---------------------|
| a. Hexamethyldisiloxane (HMDS) | supplied by DCC |
| b. ¹³ C-Hexamethyldisiloxane (¹³ C-HMDS) | supplied by DCC |
| c. Tetrahydrofuran anhydrous 99.9% (THF) | supplied by Aldrich |
| d. Magnesium sulfate (MgSO ₄) | supplied by Rater |

2. Equipment

GC/MS	HP 6890 HP Chemstation Software	Hewlett Packard
Column	HP-5 MS 30 m x 0.25 mm ID, 0.25 μm film thickness	Hewlett Packard
Centrifuge	GH 3.8 rotor	IEC Contra-8R Centrifuge Beckman GS-6R
Vortexer		VWR Multi-Tube Vortexer
Autosampler vials	2 mL crimp top, clear glass	Hewlett Packard
Limited volume inserts	100-μL glass	Alltech
Aluminum crimp caps	teflon-lined red/orange butyl rubber septa	Hewlett Packard
Extractant collection vials and caps	glass – PTFE lined	Alltech
Round bottom vial	1.7-mL crimp top, clear glass	Alltech
Scissors	stainless steel surgical	

Note: equivalent equipment may be substituted for any of the above.

3. General

An analytical balance shall be used for gravimetric preparation of all standards and samples on a weight of solute per weight of solution basis. Volumetric addition of QC spike can be made if syringe test (at least 5 replicate weights of 20- μ L aliquots of QC spike solution into GC autosampler vial). The average weight per volume will be used to calculate expected concentration of QC spikes.

PREPARATION OF REAGENTS

1. Internal Standard Stock Preparation

Accurately weigh and record to the nearest 0.1 mg in a glass vial previously capped and tared, approximately 15.0 mg of M₄Q or ¹³C-HMDS (IS). Add approximately 18 mL of THF, obtain final weight of solution and mix well.

2. Internal Standard Working Solution

The internal standard working solution (ISTD) which is added to all working solvent standards and QC matrix samples consists of THF containing (IS). To prepare 100-mL of internal standard solution, weigh and record to the nearest 0.1 mg, approximately 6 mL of internal standard stock solution in a suitable glass vial. Add approximately 100 mL of THF, cap and vortex gently for 30 seconds. Obtain the final weight of the standard solution.

3. Standard Preparation (STD)

3.1 STD STK

In a suitable glass vial, previously capped and tared, weigh and record to the nearest 0.1 mg, approximately 18.0 mg of HMDS. Add approximately 15 mL of THF, cap and vortex gently for 30 seconds at motor speed 2. Obtain the final weight of standard and THF.

3.2 STD STK 1

In a suitable glass vial, previously capped and tared, weigh and record to the nearest 0.1 mg approximately 1 mL of STD STK (above). Add approximately 9 mL of THF, cap and vortex gently for 30 seconds at motor speed 2. Obtain the final weight of standard and THF.

3.3 STD STK 2

In a suitable glass vial, previously capped and tared, weigh and record to the nearest 0.1 mg approximately 0.25 mL of STD STK 1 (above). Add approximately 10 mL of THF, cap and vortex gently for 30 seconds at motor speed 2. Obtain the final weight of standard and THF.

4. QC Stock Solution Preparation

4.1 QC STK

In a suitable glass vial, previously capped and tared, weigh and record to the nearest 0.1 mg, approximately 15.0 mg of HMDS. Add approximately 10 mL of THF, cap and vortex gently for 30 seconds at motor speed 2. Obtain the final weight of standard and THF.

4.2 QC STK 1

In a suitable glass vial, previously capped and tared, weigh and record to the nearest 0.1 mg approximately 0.60 mL of QC STK (see section 4.1). Add approximately 10 mL of THF, cap and vortex gently for 30 seconds at motor speed 2. Obtain the final weight of standard and THF.

4.3 QC STK 2

In a suitable glass vial, previously capped and tared, weigh and record to the nearest 0.1 mg approximately 0.10 mL of QC STK (see section 4.1). Add approximately 10 mL of THF, cap and vortex gently for 30 seconds at motor speed 2. Obtain the final weight of standard and THF.

NOTE:

Record all STD STK and QC STK preparation information on an appropriate form.
Reagent volumes and weights may be adjusted as long as exact concentrations are known.

STD CURVE PREPARATION

Place approximately 250 mg of $MgSO_4$ into round bottom glass vials. Prepare solvent standards with the appropriate standard stock solution and internal standard solution according to the following table. Obtain the weight of the aliquot of standard stock and the aliquot of internal standard solution placed in the vial containing $MgSO_4$ all to the nearest 0.1 mg. Add the appropriate volume of THF to obtain final volume of approximately 1-mL. Vortex each standard approximately 15 seconds. Dry (with $MgSO_4$) at least 1 hour. Centrifuge each standard at a setting of 2800 rpm for approximately 15 min. Place an aliquot of each standard in a limited volume insert in an autosampler vial and analyze by GC/MS.

TABLE 1: STD Curve

Standard ID	Volume of STD STK (mL)	STD STK used	Volume of Internal standard solution (mL)	Total Volume (mL)	Approximate amount of IS added (ng)	Approximate amount HMDS added (ng)
Std A	0	none	0.5	1.00	25000	0
Std B	0.015	STD STK 2	0.5	1.00	25000	50
Std C	0.030	STD STK 2	0.5	1.00	25000	100
Std D	0.060	STD STK 2	0.5	1.00	25000	200
Std E	0.150	STD STK 2	0.5	1.00	25000	500
Std F	0.290	STD STK 2	0.5	1.00	25000	1000
Std G	0.015	STD STK 1	0.5	1.00	25000	2000

Std H	0.030	STD STK 1	0.5	1.00	25000	4000
Std I	0.060	STD STK 1	0.5	1.00	25000	8000
Std J	0.120	STD STK 1	0.5	1.00	25000	16000
Std K	0.180	STD STK 1	0.5	1.00	25000	24000
Std L	0.030	STD STK	0.5	1.00	25000	36000
Std M	0.060	STD STK	0.5	1.00	25000	72000
Std N	0.120	STD STK	0.5	1.00	25000	144000
Std O	0.120	STD STK	none	1.00	0	144000

STD (ng) = Concentration STD STK or STD STK1 or STD STK 2) (ng/g) X Wt. of STD (g)

Standard stock solutions and working standard solutions are stable at (5 ± 4°C) for up to 9 days.

QC SAMPLE PREPARATION

Prepare each matrix spike in duplicate according to the table below. Place appropriate matrix (~250 mg blood, kidney) into the appropriate vials (blood vials contain 5 glass beads, 3 mm diameter) and spike with appropriate QC stock solution as shown below. Syringe test: Using QC STK and syringe make at least 5 replicate weights of 20-µL aliquots of QC spike solution into an autosampler vial and record weight. The average weight per µL will be used to determine the expected concentration of the QC samples.

Table 2: QC Matrix Samples (blood)

Standard ID	Volume of STD STK (mL)	STD STK used	Volume of Internal standard solution (mL) (1st extract)	Total Volume ^a (mL)	Approximate amount of IS added (ng)	Approximate amount HMDS added (ng)
QC-0	0.000	none	0.5	1.00	25000	0
QC-1	0.020	QC STK 2	0.5	1.00	25000	300
QC-2	0.015	QC STK	0.5	1.00	25000	22500

^a Total volume is based on 1 extraction at approximately 0.5 mL of internal standard solution (weight obtained) and an additional extraction at approximately 0.5 mL of THF.

Table 3: QC Matrix Samples (kidney)

Standard ID	Volume of STD STK (mL)	STD STK used	Volume of Internal standard solution (mL) (1st extract)	Total Volume ^a (mL)	Approximate amount of IS added (ng)	Approximate amount HMDS added (ng)
QC-0	0.000	none	3.0	6.00	25000	0
QC-1	0.020	QC STK 1	3.0	6.00	25000	1700
QC-2	0.020	QC STK	3.0	6.00	25000	30000

^a Total volume is based on 1 extraction at approximately 3.0 mL of internal standard solution (weight obtained) followed by addition of approximately 3.0 mL of THF.

QC samples are then extracted with THF according to the Sample Preparation Section below starting with step 1.2, (do not add blood in step 1.3) or 2.3 depending on the matrix used.

SAMPLE PREPARATION

1. Blood Extraction

- 1.1. Add ~5 glass beads to extraction vial, use septa cap for vial.
- 1.2. Add ~0.5 mL of internal standard solution and obtain weight.
- 1.3. Add approximately 150-250 mg of blood to vial, obtain weight, and vortex at least 5 minutes.
- 1.4. Centrifuge at least 5 minutes at 2800 rpm and transfer the supernatant to a new pre-weighed glass vial.
- 1.5. Add 0.5 mL of THF to the extraction vial for a second extraction and vortex at least 5 minutes.
- 1.6. Centrifuge at least 5 minutes at 2800 rpm and transfer the supernatant to the same glass vial containing the first extract. Obtain weight of combined extracts. (note: extractant weight only needed if extractant will be used for Radiochemical analysis.)
- 1.7. Add approximately 250 mg of $MgSO_4$ to glass round bottom vials. Transfer ~600 μL of the above combined extracts to the glass round bottom vials, cap and vortex for approximately 15 seconds, and allow to dry at least 1 hour.
- 1.8. Centrifuge the samples for at least 15 minutes at 2800 rpm.
- 1.9. Transfer an aliquot of the supernatant to a low volume insert in a GC autosampler vial.

2. Kidney

- 2.1. Obtain vial containing (weight previously obtained) tissue sample. (If frozen allow to thaw on ice.)
- 2.2. Remove from ice, wipe water from outside of vial and obtain tare weight of vial (or tare balance) containing sample.
- 2.3. Remove cap, add 3.0 mL of internal standard solution, obtain weight, and add additional 3.0 mL of THF. Note: The order of addition of internal standard solution and sample may be reversed as when extracting directly after collection as long as the weights are obtained.
- 2.4. With scissors, cut tissue into small pieces to improve surface area contact with THF.
- 2.5. Vortex at least 5 minutes.

- 2.6. Transfer the supernatant to a new pre-weighed glass vial. Obtain weight of extract. (note: extractant weight only needed if extractant will be used for Radiochemical analysis.)
- 2.7. Add approximately 250 mg of MgSO₄ to glass round bottom vials. Transfer ~600 µL of the above extracts to the glass round bottom vials, cap and vortex for approximately 15 seconds, and allow to dry at least 1 hour.
- 2.8. Centrifuge the samples for at least 15 minutes at 2800 rpm.
- 2.9. Transfer an aliquot of the supernatant to a low volume insert in a GC autosampler vial.

Note: Once samples are in extraction solvent, samples are stable up to 9 days at a temperature no warmer than 5±4 degrees C.

ANALYSIS

Samples shall be analyzed by GC/MS using the instrument parameters shown in Table 5.

Injection and oven ramp parameters may be edited as long as the same parameters are used for solvent standards, QC samples and samples for a given analysis.

Table 5. Analysis Parameters

Instrument:	Hewlett Packard 6890 Gas Chromatograph/Mass Selective Detector		
Column:	Hewlett Packard HP-5MS 30 m x 0.25 mm ID with 0.25 um film thickness		
Carrier Gas:	Helium, initial pressure 7.5 psi, 1.0 mL/min, constant flow on		
Injection:	temperature 250°C, splitless, 1 µL injection		
Oven Ramp:	Initial 50°C for 2.5 min, ramp to 230°C at 40°C/min, hold for 1 min, total run time 8.0 min.		
Detection:	MSD transfer line temperature 280°C		
Quantitation ions:	HMDS:	147 m/z at 100msec	
	¹³ C-HMDS	152 m/z at 100msec	

Single injection analysis of each sample is sufficient.

Sample Analysis Order (Example)

Analyze each matrix in separate analysis runs. Separate analysis runs may occur on the same day.

Solvent Blank
Solvent Internal Standard Blank (3 injections)
Solvent Calibration Standards, (Low to High)
Solvent Blank
QC Samples (Low to High)
Solvent Blank
Solvent Standard
Solvent Blank

10 Samples or Less
Solvent Standard
Solvent Blank

Repeat this Solvent standard and Sample analysis pattern until all samples are analyzed.

DATA ANALYSIS

This section describes the calculations for the calibration of the GC/MS and the method to determine the amount of HMDS per gram of blood or kidney.

All calculations for routine sample analysis shall be performed using a Microsoft Excel spreadsheet (a spreadsheet which has been prepared for a specific application and has been confirmed by an independent review to perform calculations as defined; subsequent uses of the spreadsheet require 100% check of all entered data).

1. Instrument Calibration Calculations

Calibration of the mass spectrometer is performed using HMDS concentrations expressed in terms of μg HMDS. The nominal concentrations of calibration standards are shown in Table 1. The standard curve may be split into multiple ranges depending on the range of the instrument as long as at least 4 standards make up a range.

HMDS Calibration Equation

Calculate a suitable regression equation, using a suitable regression program (e.g. linear or power), for HMDS where $x = \text{amount ratio } (\mu\text{g HMDS})/(\mu\text{g } ^{13}\text{C-HMDS})$ and $y = \text{peak area response ratio for the calibration standards from the GC/MS analysis}$. Enter the resulting slope (m) and y -intercept (b) from each equation into the spreadsheet.

$y = mx + b$, where $y = \text{peak area response ratio}$ and $x = \mu\text{g HMDS}/\mu\text{g } ^{13}\text{C-HMDS}$
alternatively a power regression may be used, $y = mx^b$

2. Calculation of HMDS Concentrations in Samples ($\mu\text{g HMDS}/\mu\text{g } ^{13}\text{C-HMDS}$)

The concentration of HMDS in a sample extract is calculated once the slope (m) and y -intercept (b) have been entered into the spreadsheet. The concentration ($\mu\text{g HMDS}$) of HMDS in a sample extract is calculated by substitution of the peak area response ratio for y into the linear equation generated from calibration standards and solving for x , ($x = (y-b)/m$) or ($x = (y/m)^{(1/b)}$).

3. Calculation of HMDS Concentrations in Samples ($\mu\text{g HMDS}/\text{g matrix}$)

Calculation of HMDS concentration ($\mu\text{g}/\text{g}$) in sample matrix is as follows:

$$\text{HMDS } (\mu\text{g}/\text{g}) = \frac{((\text{HMDS } (\mu\text{g})/^{13}\text{C-HMDS } (\mu\text{g})) \times ^{13}\text{C-HMDS } \mu\text{g added})/\text{Sample matrix weight (g)}}$$

DATA ACCEPTANCE

1. Calibration Acceptance Criteria

Agreement between the analyzed and prepared concentrations of HMDS in the calibration standards must be achieved to prove conformance to the linear calibration model. The percent relative error, calculated by the qualified spreadsheet, shall be used to prove conformance and is calculated by subtracting the analyzed concentration from the prepared concentration, and then dividing by the prepared concentration and multiplying by 100. The percent relative error shall be within 20% for every calibration standard analyzed for the calibration to be acceptable. Calibrations that do not meet these requirements shall be brought to the attention of the Study Director (or designee). Exceptions to this calibration acceptance criteria shall be made if all samples are bracketed by calibration standards that did meet the calibration acceptance criteria. The solvent standards that are run intermittently throughout the run are to be within 20% for the run to be accepted. Any samples run before a standard meeting this acceptance criteria will be accepted. If any standards do not meet this acceptance criteria, any samples run after the standard will be evaluated by the bioanalytical supervisor in order to determine if they will be accepted.

ATTACHMENT B

Individual QC and Sample Results
Individual Quality Control (QC) Sample Results During Study Sample Analysis

QC Name		Expected HMDS µg	Observed HMDS µg	%Relative Error (Accuracy)	% Recovery	Average % Recovery	Average % Relative Error (Accuracy)
Blood	QC 0-a	0.0	BLQ				
Blood	QC 0-b	0.0	BLQ				
Blood	QC 1-a	0.209	0.0429	-79.4%	20.6%	21.0%	-79.0%
Blood	QC 1-b	0.209	0.0448	-78.5%	21.5%		
Blood	QC 2-a	16.1	14.9	-7.31%	92.7%	86.4%	-13.6%
Blood	QC 2-b	16.1	12.9	-19.8%	80.2%		
Kidney	QC 0-a	0.0	BLQ				
Kidney	QC 0-b	0.0	BLQ				
Kidney	QC 1-a	1.24	0.338	-72.7%	27.3%	27.0%	-73.0%
Kidney	QC 1-b	1.24	0.331	-73.2%	26.8%		
Kidney	QC 2-a	21.5	20.3	-5.55%	94.5%	90.4%	-9.56%
Kidney	QC 2-b	21.5	18.6	-13.6%	86.4%		

BLQ = Below Limit of Quantitation

QC 1-a and QC 1-b for both matrices yielded analyzed concentrations which were >70% below the targeted concentrations. The remaining matrix spikes (QC 2-a and b) resulted in analyzed concentrations within the range of the analyzed samples (both blood and kidney). Therefore, the recoveries of the QC 2 spikes will be used to demonstrate the success of the overall parent analysis.

Individual Sample Results

Sample ID	Rad				Rad			
	Parent µg/g Blood	Extract µg eq/g Blood	Rad Pellet µg eq/g Blood	Total Rad µg eq/g Blood	Parent µg/g Kidney	Extract µg eq/g Kidney	Rad Pellet µg eq/g Kidney	Total Rad µg eq/g Kidney
4094	13.9	38.7	13.5	52.1	137	220	46.1	266
4095	16.1	38.6	10.5	49.0	146	210	40.1	250
4096	13.1	36.8	12.3	49.1	127	208	42.5	250
4097	16.6	37.7	13.1	50.8	137	208	51.4	260
4098	11.7	35.2	12.9	48.2	111	179	40.7	220
4089	12.5	30.7	11.5	42.2	118	173	34.8	208
4100	14.3	37.5	17.3	54.9	123	207	60.6	267
4101	6.88	30.4	13.7	44.1	80.8	141	30.4	172
4102	11.0	29.2	11.5	40.6	93.8	136	29.2	165
4103	18.9	45.5	15.1	60.7	131	206	55.1	261
Average	13.5	36.0	13.1	49.2	120	189	43.1	232
StdError	1.06	1.55	0.627	1.90	6.43	9.54	3.28	12.3

Sample ID	Rad				Rad			
	Parent µg/g Blood	Extract µg eq/g Blood	Rad Pellet µg eq/g Blood	Total Rad µg eq/g Blood	Parent µg/g Kidney	Extract µg eq/g Kidney	Rad Pellet µg eq/g Kidney	Total Rad µg eq/g Kidney
4104	11.7	27.9	11.5	39.3	39.2	69.9	15.4	85.3
4105	14.0	33.4	13.4	46.8	52.7	91.0	19.3	110
4106	16.0	36.8	11.6	48.4	64.1	106	21.5	127
4107	15.1	35.7	13.8	49.5	56.8	95.6	19.9	115
4108	13.0	27.5	14.5	42.0	46.0	74.8	14.4	89.2
4109	16.2	37.4	13.2	50.6	65.9	118	31.8	150
4110	12.5	34.9	15.0	49.9	58.4	106	27.2	134
4111	17.8	39.0	13.3	52.3	66.8	103	18.9	122
4112	17.3	43.2	17.0	60.2	70.1	124	30.4	154
4113	16.4	32.7	13.4	46.1	66.9	101	17.6	119
Average	15.0	34.9	13.7	48.5	58.7	99.0	21.6	121
StdError	0.663	1.51	0.507	1.80	3.21	5.38	1.93	7.12

Sample ID 4094 - 4103 (including 4089) are male Fischer 344 rats.

Sample ID 4104 - 4113 are female Fischer 344 rats.

BLQ = Below Limit of Quantitation

N/AP = Not Applicable

Appendix F

DOW CORNING CORPORATION HEALTH & ENVIRONMENTAL SCIENCES

CONTRIBUTING SCIENTIST REPORT

Study Title: NON-REGULATED STUDY: Study of Hexamethyldisiloxane (HMDS): Determination of the Reversible Binding of HMDS/Metabolites to Alpha 2u-Globulin in Male Fischer 344 Rats Following Oral Gavage Administration

Study Number: HES Study Number: 10461-101

Test Article: ¹⁴C- Hexamethyldisiloxane

Contributing Scientists: Lisa M. Thackery, B.S.
Senior Analytical Chemist

Study Leader: Jeanne Y. Domoradzki, Ph.D., D.A.B.T.
Toxicology Specialist

Sponsor: Dow Corning Corporation
2200 W. Salzburg Road
Auburn, MI 48611

Testing Facility: Dow Corning Corporation
Health and Environmental Sciences
2200 West Salzburg Road
Auburn, Michigan 48611

TABLE OF CONTENTS

TABLE OF CONTENTS.....	F2
APPROVAL SIGNATURES.....	F3
OBJECTIVE	F4
MATERIALS AND METHODS.....	F4
1. Method Development.....	F4
2. Study Sample Collections, Processing and Analysis.....	F5
3. Parameters Evaluated.....	F6
4. Statistical Analysis.....	F6
RESULTS AND DISCUSSION.....	F7
SUMMARY	F8
ARCHIVE.....	F8
REFERENCES	F8

LIST OF TABLES

Table 1 - Peak Area Percent Summary of Alpha 2u-globulin from HIC HPLC/DAD Analysis.....	F9
Table 2 - Peak Area Percent Summary of ¹⁴ C-HMDS and/or Metabolites from HIC HPLC/RAD Analysis.....	F9

LIST OF FIGURES

Figure 1 - Representative DAD Chromatogram of Male Kidney Cytosol Profile	F10
Figure 2 - Representative DAD Chromatogram of Female Kidney Cytosol Profile.....	F10
Figure 3 - Representative DAD Chromatogram of Male Kidney Cytosol Profile after Incubation with DMSO (Negative Control)	F11
Figure 4 - Representative DAD Chromatogram of Male Kidney Cytosol Profile after Incubation with D-Limonene Oxide	F11
Figure 5 - Representative RAD Chromatogram of Male Kidney Cytosol Profile	F12
Figure 6 - Representative RAD Chromatogram of Female Kidney Cytosol Profile.....	F12
Figure 7 - Representative RAD Chromatogram of Male Kidney Cytosol Profile after Incubation with DMSO (Negative Control)	F13
Figure 8 - Representative RAD Chromatogram of Male Kidney Cytosol Profile after Incubation with D-Limonene Oxide	F13
Figure 9 - Dot Blot of Representative Male versus Female Kidney Cytosol Fractions Collected From HIC	F14

LIST OF ATTACHMENTS

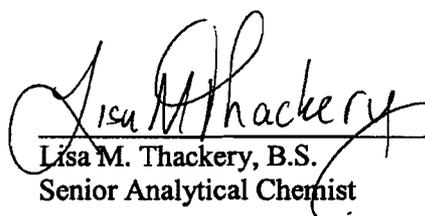
ATTACHMENT A - Procedure for Determining the Interaction between Hexamethyldisiloxane (HMDS) and α 2u-globulin, a Male Rat Specific Protein (Fisher 344 Rat Kidney – Oral).....	F15
--	-----

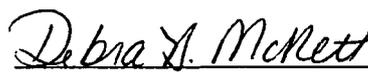
APPROVAL SIGNATURES

Report Title: **NON-REGULATED STUDY:** Study of
Hexamethyldisiloxane (HMDS): Determination of
the Reversible Binding of HMDS/Metabolites to
Alpha 2u-Globulin in Male Fischer 344 Rats
Following Oral Gavage Administration

Submitted by: Dow Corning Corporation
Health and Environmental Sciences (HES)
2200 W. Salzburg Rd.
Auburn, Michigan 48611

Approved by:

 30-Aug-2007
Lisa M. Thackery, B.S.
Senior Analytical Chemist

 30-aug-2007
Debra A. McNett, B.S.
Bioanalytical Supervisor

 30 August 2007
Jeanne Y. Domoradzki, Ph.D., D.A.B.T.
Toxicology Specialist
Study Leader

 30-Aug-2007
Roy A. Campbell, B.S.
Health and Environmental Sciences
HES Group Manager - Chemistry and
Environmental

OBJECTIVE

The objective of this study was to evaluate alpha 2u-nephropathy induction and the reversible binding of alpha 2u-globulin to HMDS and/or metabolites in male rat kidneys following oral gavage administration of HMDS in a corn oil vehicle to male and female (control) animals for 28 days over a target dose level range of 10 to 1000 mg/kg body weight. The interaction between HMDS and/or metabolites with alpha 2u-globulin in male rat kidneys was qualitatively evaluated from the 1000 mg/kg dose group only; Group 5.

MATERIALS AND METHODS

1. Method Development

A method was developed to qualitatively assess the interaction between HMDS and/or metabolites to alpha 2u-globulin in male rat kidney cytosol. The method was based upon the ability to resolve the alpha 2u-globulin protein from most other kidney proteins by HPLC (High Pressure Liquid Chromatography) using a HIC (Hydrophobic Interaction Chromatography) column without completely denaturing the protein and preserving biological activity of the protein. The mobile phase consisted of a decreasing salt gradient. The proteins are retained by increasing their hydrophobic interaction with the stationary phase at high salt concentrations and eluted by lowering the salt concentration. UV detection using a diode array detector (DAD) was used to visualize the protein separation followed by in-line radiometric detection to detect the radioactivity (metabolites and/or parent analyte) bound to the alpha 2u-globulin based on co-elution. Since alpha 2u-globulin is a male specific protein, male kidney cytosol versus female kidney cytosol was analyzed to confirm the presence and resolution of this protein.

Fractions collected from kidney cytosol proteins resolved by the HIC method were taken through a dot blot procedure to verify the identification of alpha 2u-globulin peak. Protein fractions were bound to a nitrocellulose membrane followed by immunodetection with purified Anti-rat alpha 2u-globulin Goat IgG. The InVitrogen Western Breeze® Chromogenic Western Blot Immunodetection Kit was used to detect primary antibodies immobilized by the alpha 2u-globulin.

Additionally, due to its known affinity to alpha 2u-globulin (1), D-limonene oxide was incubated with kidney cytosol samples to displace bound HMDS and/or metabolite to the protein to indicate reversible binding.

The method was slightly modified from the previous method described for HES study 10120 (2).

2. Study Sample Collections, Processing and Analysis

The test substance (unlabeled) in corn oil vehicle at the targeted dose was introduced by gavage directly into the stomachs of the study animals for 27 consecutive days. On day 28, the test substance (^{14}C -labeled, 0.414mCi/g) in corn oil vehicle at the targeted dose was introduced by gavage directly into the stomachs of the study animals. Kidneys, one kidney from each animals, were collected 2-hours following the day 28 dose administration, frozen on dry ice, and were stored at $-80^{\circ}\text{C}\pm 10^{\circ}\text{C}$ until processing. The following procedures are further detailed in **Attachment A**, "Procedure for Determining the Interaction between Hexamethyldisiloxane (HMDS) and $\alpha 2\text{u}$ -globulin, a Male Rat Specific Protein" with slight revisions described below.

Kidney Cytosol Preparation

A single kidney from each animal in Group 5 was thawed on ice. Each kidney was homogenized in chilled 20mM Tris buffer solution using Caframo RZR 50 stirrer. Homogenates were transferred to chilled polyallomer Quick-Seal® bell-top centrifuge tubes (Beckman). Additional Tris buffer may have been added to each tube to bring volume to top of tube per manufacture's instructions and the tubes were heat sealed. Samples were then ultracentrifuged (Beckman XL-90) with parameters set at 116,000g, 4°C for 1 hour. The tops of the sealed tubes were cut off and the supernatant removed for analysis. Remaining sample was stored at $-80^{\circ}\text{C}\pm 10^{\circ}\text{C}$.

HPLC HIC Analysis

Qualitative analysis was performed using HPLC (Agilent 1200 series) with a diode array detector (DAD) at 221nm and an in-line radiochemical detector (RAD). Resolution of the kidney cytosol proteins were achieved using a ProPac® HIC-10 column (Dionex) and co-elution of the alpha 2u-globulin protein with ^{14}C -labeled material was evaluated.

D-Limonene Oxide Competition

On the same day as initial kidney cytosol preparation and HPLC HIC analysis, two additional aliquots (750 μL) of each cytosol sample were transferred to HPLC vials. Based on radioactivity analysis determined in **Appendix E**, where total radioactivity expressed as total burden in each kidney ($\mu\text{g eq}$), a D-limonene oxide solution was prepared in dimethyl sulfoxide (DMSO) to deliver greater than 10-fold higher $\mu\text{g eq}$ (in less than 1% total solution) to compete with the bound radioactivity. 7.5 μL of the D-limonene oxide solution ($\sim 33\text{mg/mL}$) was added to one aliquot of sample and 7.5 μL of

DMSO only was added to the other aliquot as a negative control. The treated and control sample vials were immediately crimp-capped and incubated for 1 hour at 37°C. The samples were then analyzed by the HPLC HIC method to qualitatively assess whether ¹⁴C-labeled HMDS and/or metabolites were reversibly bound to alpha 2u-globulin.

Dot Blot

A single kidney cytosol sample from male rat kidney and one from female rat kidney were taken from -80°C±10°C storage and thawed. These samples were injected on to the HPLC to resolve the kidney proteins by the HIC method. Eluent fractions were collected after the diode array detector at the retention time range of the alpha 2u-globulin peak. A blunt tipped syringe was used to apply 5, 10, 20, and 40µL of the eluent sample to a dry nitrocellulose membrane as well as 10 and 40µL 5mg/mL chymotrypsinogen A and ribonuclease A (negative controls to rule out non-specific protein binding; LMW Gel Filtration Calibration Kit from Amersham Biosciences). The blotted proteins were allowed to bind to the nitrocellulose for at least 20 minutes and then any excess liquid was blotted off the membrane using a Kimwipe. Purified anti-rat α_{2u}-globulin antibody goat IgG (AF586, R&D Systems) were used for immunodetection of the proteins collected from the HIC fractions. The WesternBreeze® Chromogenic Immunodetection Kit (WB7107, Invitrogen) was used to detect the primary antibodies that were immobilized by any present alpha 2u-globulin on the blot.

3. Parameters Evaluated

The alpha 2u-globulin protein elution from HIC HPLC was monitored at 221nm by DAD. Percent peak areas of the total peak areas at the elution time range for alpha 2u-globulin (approximately 9-11 minutes) were reported for all male and female kidney samples. Percent peak areas of the total peak areas at the elution range for alpha 2u-globulin were also reported for DMSO control and D-limonene oxide treated male kidney samples and a subset of the female kidney samples. Additionally, percent of total radioactivity was measured for the peak co-eluting with the alpha 2u-globulin protein. Dot blot verification of the peak identified as alpha 2u-globulin was performed on one male representative sample versus one female representative sample in order to observe a visual positive or negative chromogenic event.

4. Statistical Analysis

Numerical data obtained during the conduct of the study were recorded for qualitative comparison only. Group mean values were processed Microsoft

Excel™ Version 11. Rounding differences in tables may occur due to the calculation of numbers using more significant figures than are shown.

RESULTS AND DISCUSSION

The individual results from the HIC HPLC/DAD analysis is presented in **Table 1** which expresses the amount of alpha 2u-globulin per sample as the percent peak area of the total peak area for each male and female kidney sample. Since only males produce alpha 2u-globulin, the value reported for females represents the background protein (as the column is most likely overloaded) at the approximate time range alpha 2u-globulin was observed in the male samples. Representative male and female DAD chromatograms are shown in **Figures 1 and 2** respectively. On average, the alpha 2u-globulin peak was observed to be 27% of the total peak area of the male kidney samples while the integrated percent peak area in that approximate elution time range for the female kidney samples was 10%.

Table 1 also shows the DAD results alpha 2u-globulin percent peak area of the total peak area for DMSO control and D-limonene oxide treated male kidney samples and a subset of the female kidney samples. **Figures 3 and 4** are representative DAD chromatograms. The remaining female samples were not taken through the D-limonene oxide treatment since again, only males produce alpha 2u-globulin. Alpha 2u-globulin remained, on average, as approximately 26% and 28% of total peak area through the incubation with negative control (DMSO) and D-limonene oxide treatment respectively. In general this indicates the alpha 2u-globulin protein was not significantly degraded by the addition of DMSO or D-limonene oxide, or during the 1 hour incubation process, or while sitting in the sample queue before injection.

Radioactivity co-eluting with the alpha 2u-globulin peak was an average of 54% of the total radioactivity per male kidney sample (**Table 2**). **Figure 5** is a representative chromatogram from the radiometric detector (RAD) from a male kidney sample. In addition to the radioactive peak that co-eluted with alpha 2u-globulin peak, another radioactive species was observed at approximately 3 minutes that may be associated with other earlier eluting proteins in the male kidney cytosol samples. If this species is not protein associated, it must be a free aqueous soluble metabolite. Female kidney samples demonstrated no co-elution at the later retention time where alpha 2u-globulin elutes, as expected, but the early eluting radioactive species around 3 minutes was still observed at low levels (**Figure 6**).

In general, slight losses of bound ¹⁴C-HMDS and/or metabolites were observed in the negative control where DMSO only was incubated with the male kidney samples (**Table 2, Figure 7**). However, the D-limonene oxide treated samples demonstrated complete loss of bound radioactivity at the same elution retention time as alpha 2u-globulin and an appearance of an additional radioactive peak at approximately 4 minutes was observed

(Figure 8). Thus, the D-limonene was able to compete with bound ^{14}C -HMDS and/or metabolites to the alpha 2u-globulin protein and demonstrate the binding was reversible. The results of the dot blot to confirm the identity of the alpha 2u-globulin peak is shown in Figure 9. The eluent fraction collected for the male kidney sample clearly demonstrated a positive chromogenic response and as expected, the fraction collected from the female kidney sample did not elicit a response. This indicates that antibodies are specifically bound to alpha 2u-globulin from the male on the blot. Additional control proteins blotted to the nitrocellulose also did not demonstrate a chromogenic endpoint and thus further support that the primary and secondary antibodies incubated with the nitrocellulose did not bind to protein nonspecifically.

SUMMARY

A selective analytical method was developed to qualitatively assess the interaction between HMDS and/or metabolites to alpha 2u-globulin in male rat kidney. The method was then used to determine that reversible binding of ^{14}C -HMDS and/or metabolite to the alpha 2u-globulin protein does occur in male rat kidney following an oral gavage administration of ^{14}C -HMDS in corn oil at 1000 mg HMDS/kg bw.

ARCHIVE

All raw data associated with samples analysis are archived at Dow Corning Corporation, Health and Environmental Sciences, 2200 W. Salzburg Road, Auburn, MI 48611.

REFERENCES

1. Lehman-McKeeman, L.D., Rodriguez, P.A., Takigiku, R., Caudill, D., and Fey., M.L. (1989). D-Limonene-induced male rat specific nephrotoxicity: Evaluation of the association between d-limonene and α 2u-globulin. Toxicol. Appl Pharmacol. 91, 182-192.
2. DCC Study #10210-101, Hexamethyldisiloxane: Inhalation (Nose-Only) Reversible Binding Study in Male and Female Fischer 344 Rats and a Limited Disposition Pharmacokinetic Study in Female Fischer 344 Rats at 5000 ppm.

Table 1 – Percent Peak Area Summary of Alpha 2u-globulin from HIC HPLC/DAD Analysis

Analysis Date	Sample ID Males	% Peak Area	Control % Peak Area	D-limox treated % Peak Area	Sample ID Females	% Peak Area	Control % Peak Area	D-limox treated % Peak Area
6/21/2007	4094	32.45*	25.52	26.81	4104	9.46	7.99	7.86
6/21/2007	4095	23.47	23.91	25.49	4105	8.52	7.51	7.38
7/2/2007	4096	26.16	25.47	26.55	4106	9.48	9.37	8.76
7/2/2007	4097	28.49	28.73	30.50	4107	9.41	9.61	9.29
7/2/2007	4098	27.14	26.54	27.81	4108	8.51	8.67	8.21
7/6/2007	4089	31.56	27.97	28.70	4109	14.04	NA**	NA
7/6/2007	4100	27.75	24.14	25.70	4110	10.36	NA	NA
7/6/2007	4101	29.17	27.03	27.80	4111	11.10	NA	NA
7/6/2007	4102	26.31	24.78	26.59	4112	10.63	NA	NA
7/6/2007	4103	26.60	24.42	29.09	4113	9.93	NA	NA
Average		27.41	25.85	27.50		10.14	8.63	8.30

*injection volume double that of other samples (excluded from avg)

**NA - not analyzed

Table 2 – Percent Peak Area Summary of ¹⁴C-HMDS and/or Metabolites from HIC HPLC/RAD Analysis

Analysis Date	Sample ID Males	% Peak Area	DMSO Control % Peak Area	D-limox treated % Peak Area
6/21/2007	4094	56.58	61.16	13.5*
6/21/2007	4095	59.43	52.99	0.00
7/2/2007	4096	50.64	48.75	0.00
7/2/2007	4097	60.84	52.92	0.00
7/2/2007	4098	54.18	48.96	0.00
7/6/2007	4089	53.18	58.76	0.00
7/6/2007	4100	44.43	35.45	0.00
7/6/2007	4101	53.49	47.38	0.00
7/6/2007	4102	56.68	45.92	0.00
7/6/2007	4103	48.41	44.12	0.00
Average		53.79	49.64	0.00

*excluded from average calculation as integrated peak area appears to be baseline noise

Figure 1 – Representative DAD Chromatogram of Male Kidney Cytosol Profile

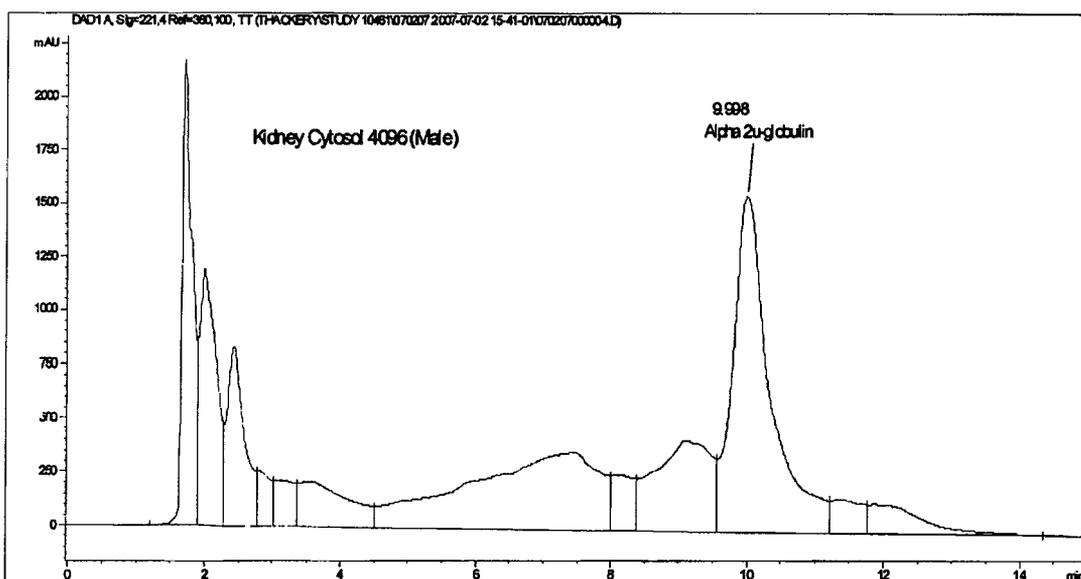


Figure 2 – Representative DAD Chromatogram of Female Kidney Cytosol Profile

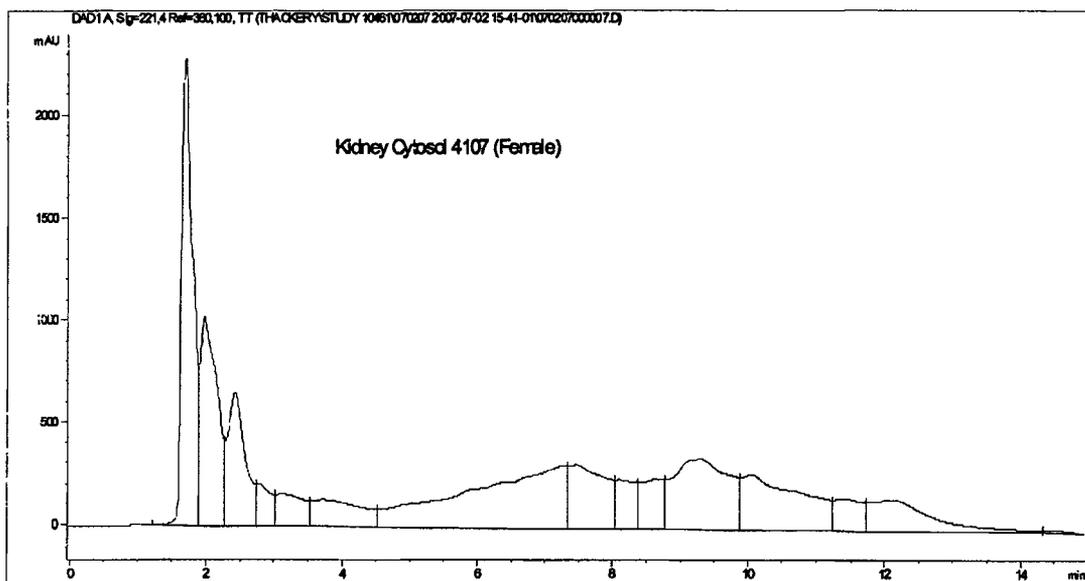


Figure 3 – Representative DAD Chromatogram of Male Kidney Cytosol Profile after Incubation with DMSO (Negative Control)

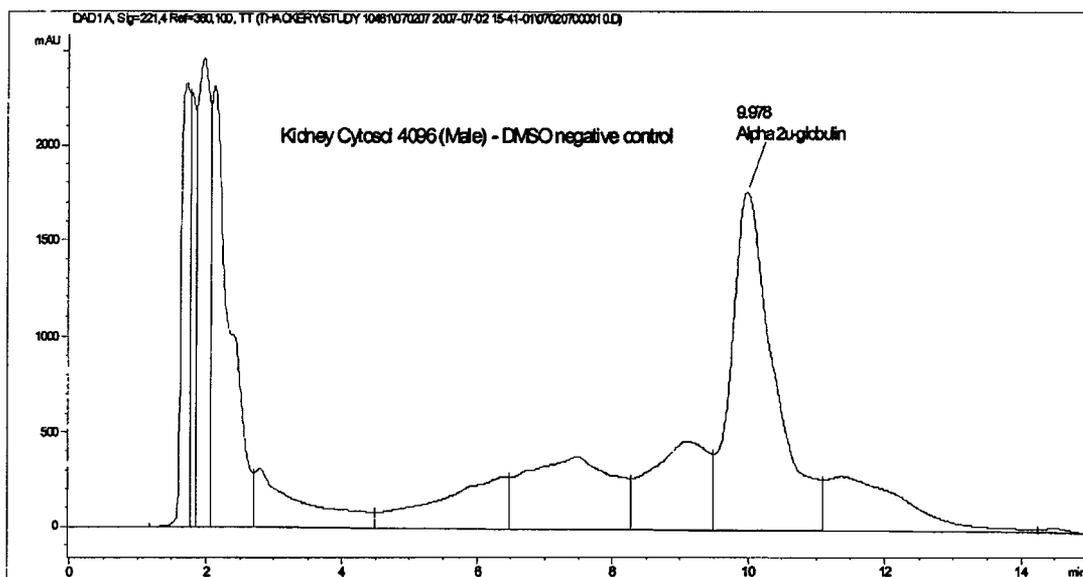


Figure 4 – Representative DAD Chromatogram of Male Kidney Cytosol Profile after Incubation with D-Limonene Oxide

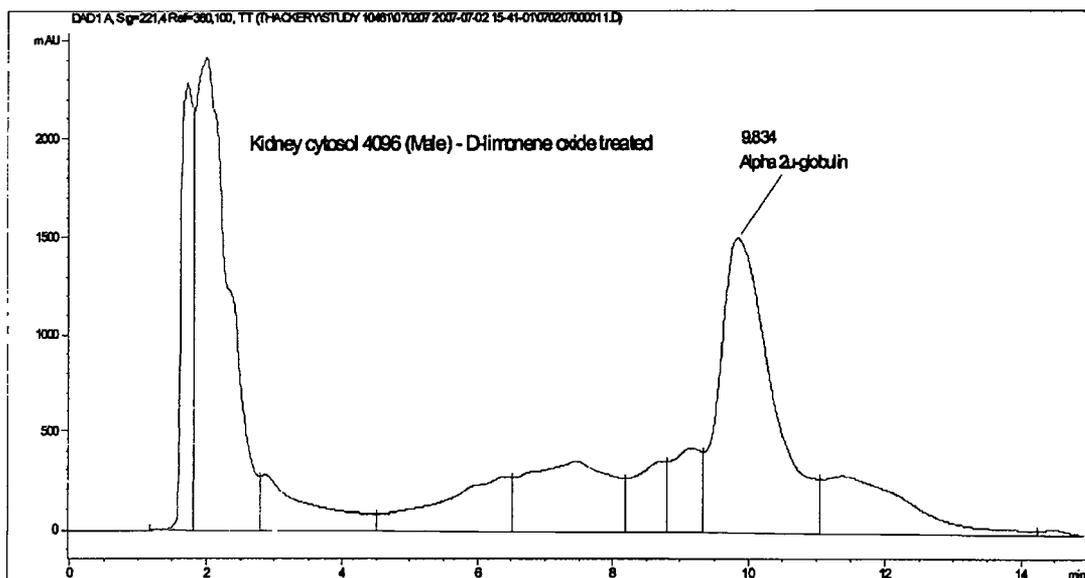


Figure 5 – Representative RAD Chromatogram of Male Kidney Cytosol Profile

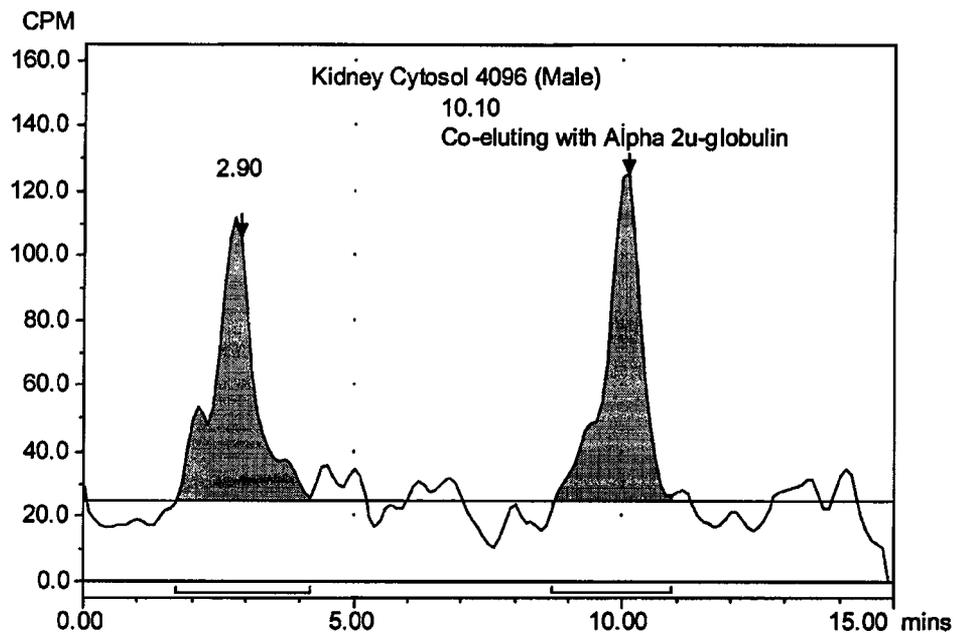


Figure 6 – Representative RAD Chromatogram of Female Kidney Cytosol Profile

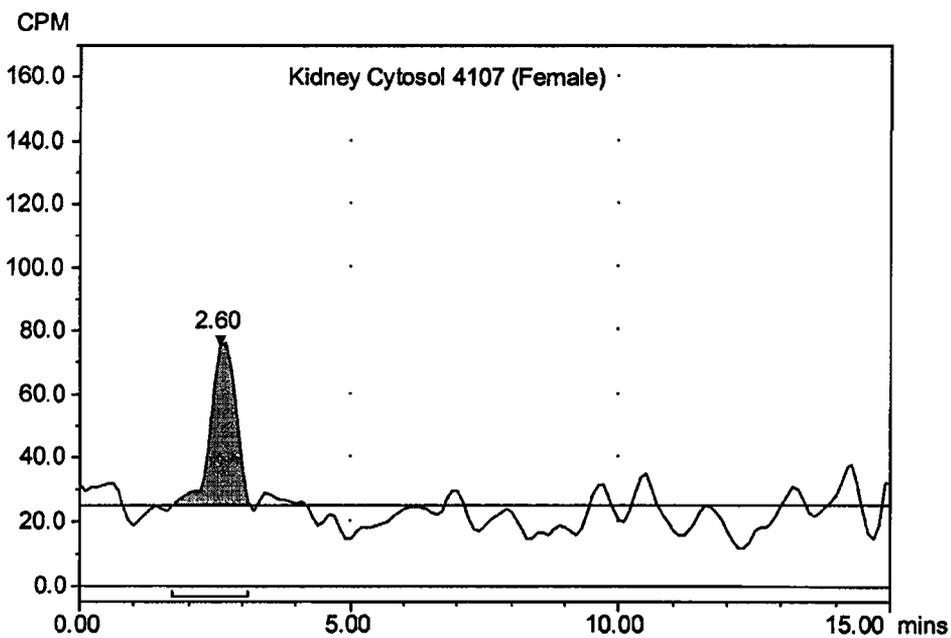


Figure 7 – Representative RAD Chromatogram of Male Kidney Cytosol Profile after Incubation with DMSO (Negative Control)

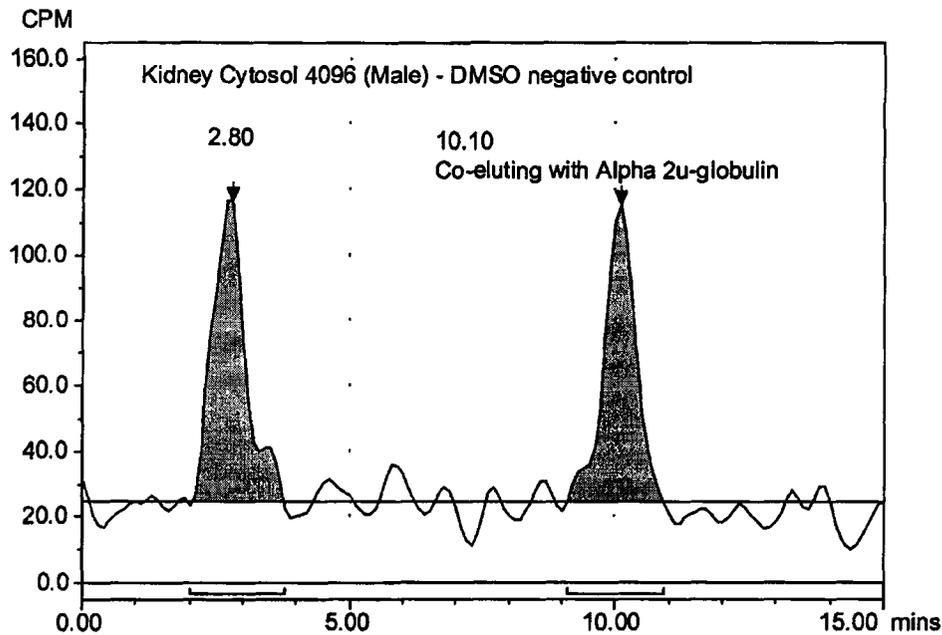


Figure 8 – Representative RAD Chromatogram of Male Kidney Cytosol Profile After Incubation with D-Limonene Oxide

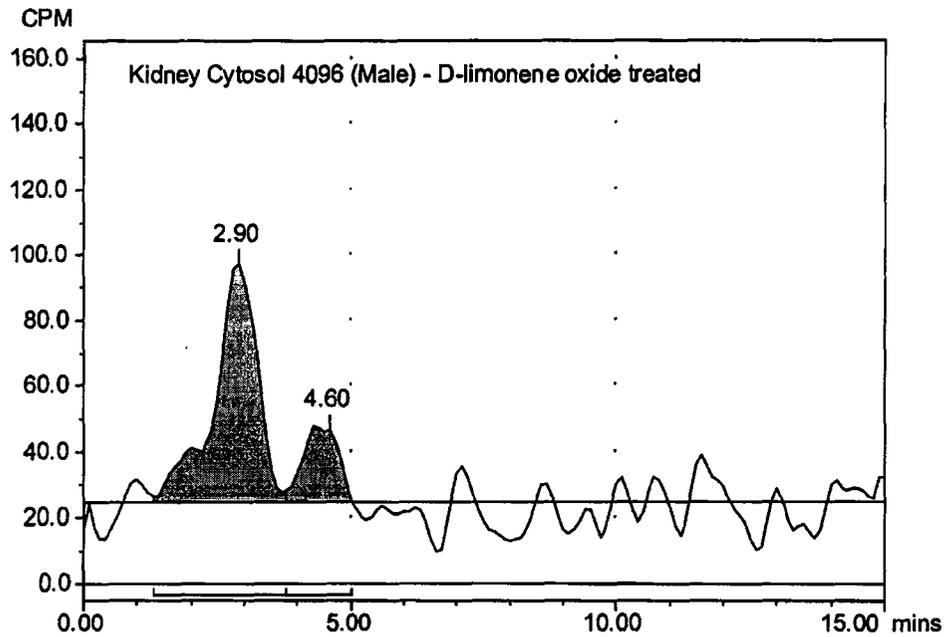
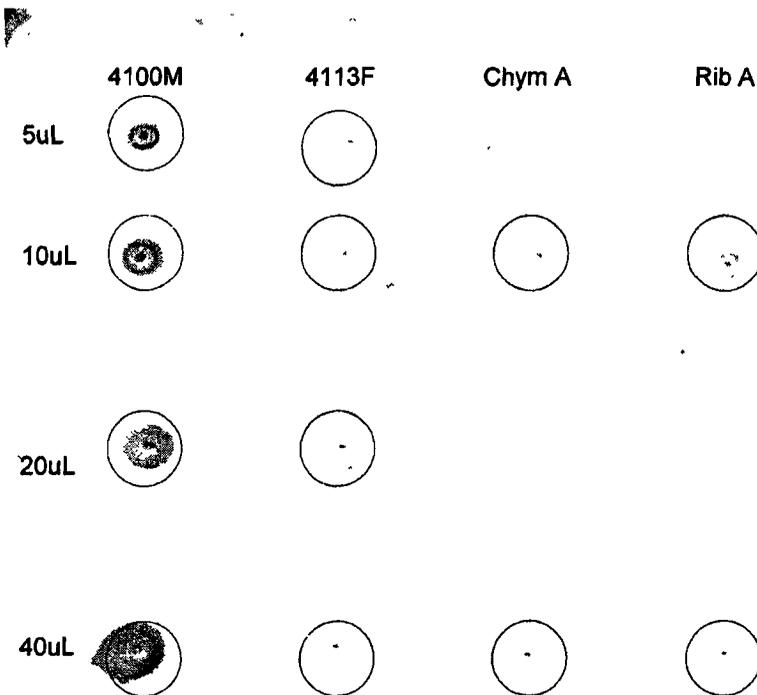


Figure 9 - Dot Blot of Representative Male versus Female Kidney Cytosol Fractions Collected From HIC



**Procedure for Determining the Interaction between Hexamethyldisiloxane (HMDS)
and α 2u-globulin, a Male Rat Specific Protein
(Fisher 344 Rat Kidney – Oral)**

Lisa M. Thackery

Lisa M. Thackery, B.S.

Date 6/17/07

Debra A. McNett

Debra A. McNett, B.S.
Bioanalytical Supervisor

Date 6/17/07

Jeanne Y. Domoradzki

Jeanne Y. Domoradzki, Ph.D., D.A.B.T
Study Director

Date 6/20/07

**Procedure for Determining the Interaction between Hexamethyldisiloxane (HMDS)
and α 2u-globulin, a Male Rat Specific Protein
(Fisher 344 Rat Kidney – Oral)**

PURPOSE

To describe a qualitative method for assessing the interaction between Hexamethyldisiloxane (HMDS) and/or metabolites to α 2u-globulin, a male rat specific protein, in rat kidneys. Procedures for various analyses may not be performed on all samples but representative groups.

MATERIALS

1. Chemicals

- | | |
|---|---------------------------|
| a. Hexamethyldisiloxane (HMDS) | supplied by Dow Corning |
| b. ^{14}C - Hexamethyldisiloxane (^{14}C -HMDS) | supplied by Dow Corning |
| c. Trizma-HCl (Tris-HCl) | supplied by Sigma |
| d. Ammonium sulfate | supplied by Sigma-Aldrich |
| e. Sodium phosphate monobasic, anhydrous (NaH_2PO_4), | supplied by Fisher |
| f. 50% Sodium hydroxide (NaOH) | supplied by Fluka |
| g. Western Breeze® Chromogenic Western Blot Immunodetection Kit | supplied by Invitrogen |
| h. Anti-rat α 2u-globulin Goat IgG (secondary Ab) | supplied by R&D Systems |
| i. LMW Gel Filtration Calibration Kit | supplied by Amersham |
| j. D-limonene oxide | supplied by Aldrich |
| k. Dimethyl sulfoxide (DMSO) | supplied by Fisher |

2. Equipment

Stirrer/homogenizer	RZR 50 stirrer	Caframo
Stirrer test tubes	10mL	Wheaton
Ultracentrifuge	XL-90	Beckman
Ultracentrifuge tubes	Quick-Seal polyallomer 16 X 38mm	Beckman
HPLC	HP 1050, Quaternary Pump	Hewlett Packard

Dow Corning Corporation
HES Study Number 10461-101
Attachment A

Detectors	DAD 1050 Radiomatic 610TR Series	Hewlett Packard Perkin-Elmer
Column	ProPac HIC 10 Column 7.8 X 75mm	Dionex
Vacuum filter funnels	0.22µm cellulose nitrate	Corning
Titer plate shaker		Lab-line
Autosampler vials	2mL crimp top, clear glass	Hewlett Packard
Limited volume	inserts 100uL glass	Alltech
Aluminum crimp caps	teflon-lined red/orange butyl rubber septa	Hewlett Packard
Extractant collection vials and caps	glass – PTFE lined	Alltech
Round bottom vial	1.7mL crimp top, clear	Alltech
pH meter 240	semimicro combination probe	Corning
1L volumetric flask		Pyrex

Note: equivalent equipment may be substituted for any of the above.

3. PROCEDURE FOR KIDNEY CYTOSOL PREPARATION

- 3.1 Prepare 20mM Tris Buffer solution
 - 3.1.1 Weigh 3.152g Tris-HCl and add to 1L volumetric flask.
 - 3.1.2 Add approximately 800mL Milli-Q water to volumetric flask and swirl to dissolve.
 - 3.1.3 Adjust pH of solution to 7.5 ± 0.1 using 50% sodium hydroxide.
 - 3.1.4 Adjust flask to volume with Milli-Q water.
 - 3.1.5 Store solution at 4-8°C for up to 1 month.
- 3.2 Thaw frozen kidney on ice in original sample container.
- 3.3 Transfer kidney to chilled glass stirrer tube, add 1mL chilled 20mM Tris buffer.
- 3.4 Homogenize kidney using homogenizer/stirrer by moving tube up and down at least 3 times, add 1mL of buffer and repeat until homogenate appears homogeneous.
- 3.5 Transfer homogenate to chilled Quickseal tube. Use an additional 2mL Tris buffer to rinse sample tube and stirrer and transfer to Quickseal tube as well.
- 3.6 Heat-seal Quickseal tubes closed according to manufacture's instructions.
- 3.7 Centrifuge samples with Ultracentrifuge parameters set at 116,000g, 4°C, 1 hour.
- 3.8 Cut top off of sealed tube and remove supernatant. An aliquot may be immediately placed into glass inserts in an autosampler vial for analysis and the remainder transferred to a suitable glass vial for storage at $\leq -20^{\circ}\text{C}$.

4. HPLC HIC (Hydrophobic Interaction Chromatography) ANALYSIS

Background: $\alpha 2\text{u}$ is resolved from most other kidney proteins using a decreasing salt gradient on the HIC column with UV detection to visualize the protein separation. Since this is a male specific protein, male kidney extract versus female kidney extract can be analyzed to confirm the presence and resolution of this protein. Radiomatic detection can then be used in-line to detect the radioactivity (metabolites and/or parent analyte) bound to the $\alpha 2\text{u}$ protein based on co-elution.

4.1 Prepare Mobile Phase A

- 4.1.1 Weigh 132.1g ammonium sulfate and add to 650mL Milli-Q water in 1L volumetric flask.
- 4.1.2 Weigh 12g NaH₂PO₄, add to volumetric flask and dissolve until all salts in solution.
- 4.1.3 Adjust pH of solution to 7.0 ± 0.1 using 50% sodium hydroxide.
- 4.1.4 Adjust flask to volume with Milli-Q water.

4.2 Prepare Mobile Phase B

- 4.2.1 Weigh 12g NaH₂PO₄, and add to 650mL Milli-Q water in 1L volumetric flask.
- 4.2.2 Dissolve until all salts in solution.
- 4.2.3 Adjust pH of solution to 7.0 ± 0.1 using 50% sodium hydroxide.
- 4.2.4 Adjust flask to volume with Milli-Q water.

4.3 Filter Mobile Phases using 0.22µm cellulose nitrate disposable vacuum filters. Mobile Phases can be stored at room temperature for up to one week.

4.4 To isolate α₂u, aliquots of the prepared kidney cytosol will be injected onto an HPLC with UV detection. Samples shall be analyzed by HPLC using the instrument parameters shown in Table 1. Injection and mobile phase parameters may be edited if necessary as long as the same parameters are used within a given analysis.

Table 1. Analysis Parameters

Instrument:	Agilent 1200 Series/DAD Detector
Column:	Dionex ProPac HIC 10 (7.8 X 75mm) 5µ
Mobile Phase	1M (NH ₄) ₂ SO ₄ , 0.1M NaH ₂ PO ₄ in Milli-Q water, pH 7.0
A:	
Mobile Phase B:	0.1M NaH ₂ PO ₄ in Milli-Q water, pH 7.0
Flow:	1.5mL/min
Gradient:	0%B 2min, to 100%B over 8min, hold 2min, to 0%B over 3min; 15min runtime, 5min post-time to re-equilibrate
Injection volume:	100µL
Detectors:	DAD store 221nm Radiomatic

Single injection analysis of each sample is sufficient. Each individual kidney processed is considered a single sample.

Sample Analysis Order (Example)

Tris Buffer Blank
Mixture of Protein Standards from LMW Gel Filtration Calibration Kit (System performance)
Tris Buffer Blank
Female kidney control
Male kidney control
10-12 additional samples
Tris Buffer Blank
Mixture of Protein Standards from LMW Gel Filtration Calibration Kit

Note: Fractions of peak identified as α 2u (present in male kidney cytosol but not female) can be collected after UV detection. Eluent from a sample injection can be used directly for identification verification by Dot-Blot (Section 6).

5. D-LIMONENE OXIDE COMPETITION

Background: Due to its known affinity to α 2u, D-limonene oxide would theoretically displace bound parent/metabolite to the protein. After sample incubation with D-limonene oxide, a decrease or loss in radioactivity co-eluting with the α 2u peak (again analyzed as seen in Section 4) would indicate a reversible binding process.

- 5.1 Prepare D-limonene oxide solution in DMSO so that 5 μ L can be added to 500 μ L kidney cytosol to target approximately 10-fold higher (μ g eq) than 14 C-HMDS/metabolites observed in kidney (based on μ g eq radioactivity total in males minus μ g eq radioactivity total in females).
- 5.2 Add 5 μ L DMSO (as a negative control) or 5 μ L D-limonene oxide solution in DMSO to 500 μ L kidney cytosol samples in HPLC vials.
- 5.3 Seal the vials with crimp caps and incubate treated and control kidney cytosol samples for 1 hour at 37°C.
- 5.4 Analyze according to HIC method described in Section 4.

6. DOT BLOT PROCEDURE

Background: Fractions collected from kidney cytosol proteins resolved by the HIC method are taken through a dot blot procedure to verify the identification of α 2u peak. Protein fractions are bound to a nitrocellulose membrane followed by immunodetection

with purified Anti-rat α 2u-globulin Goat IgG. The InVitrogen Western Breeze® Chromogenic Western Blot Immunodetection Kit is then used to detect primary antibodies immobilized by the α 2u protein.

- 6.1 Clip upper left of nitrocellulose membrane for orientation and then press nitrocellulose onto rings of a 96-well micro titer plate to create a faint circular mark. Handle nitrocellulose with forceps as much as possible, avoiding touching the working surface.
- 6.2 Using a 50 μ L blunt-tip syringe, slowly add 10, 20, and 40 μ L of protein (HIC fractions collected from male and female (negative control) kidney cytosol, and additional negative protein controls (such as those from the LMW Gel Filtration Calibration Kit to rule out non-specific binding) directly to dry nitrocellulose membrane in spaced out circular marks created in 6.1.
- 6.3 Allow proteins to soak into nitrocellulose membrane and/or spots to dry for at least 20 minutes before blocking.
- 6.4 Follow WesternBreeze® Chromogenic Immunodetection Protocol according to manufacture's instructions. Development of color indicates presence of antigen (α 2u protein).