FINAL REPORT

Guideline: EPA-OPPTS (870.1100)

Testing Facility Study No. EUF00219

Monsanto Study No. CRO-2007-324

An Acute Toxicity Study of Delta 6 Desaturase and Delta 15 Desaturase Proteins Administered by the Oral (Gavage) Route to Mice

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09 October 2008

The text below applies only to use of the data by the United States Environmental Protection Agency (US EPA) in connection with the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

The inclusion of this page in all studies is for quality assurance purposes and does not necessarily indicate that this study has been submitted to the U.S. EPA.

1. STATEMENT OF NO DATA CONFIDENTIALITY CLAIM

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA 10(d)(l)(A), (B), or (C).

"We submit this material to the United States Environmental Protection Agency specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by EPA strictly in accordance with FIFRA. By submitting this material to EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by the company notwithstanding this submission to EPA."

Company: Monsanto Company

Company Agent: _____

Title: _____

Signature: _____ Date: _____

2. COMPLIANCE STATEMENT

This study was conducted in compliance with the Good Laboratory Practice (GLP) regulations as described by the EPA (40 CFR Part 160), OECD [ENV/MC/CHEM(98)17], and JMAFF (11 Nousan No. 6283).

Jason W. Smedley 09OCT2008

Jason W. Smedley, B.S. Date
Study Director
Charles River Laboratories
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_____

Date

Sponsor/Submitter
Monsanto Company

3. QUALITY ASSURANCE STATEMENT

This study has been inspected by the Quality Assurance Unit to assure conformance with the Good Laboratory Practice (GLP) regulations promulgated by the EPA (40 CFR Part 160), OECD [ENV/MC/CHEM(98)17], and JMAFF (11 Nousan No. 6283). Reports were submitted in accordance with Standard Operating Procedures as follows:

QA INSPECTION DATES

		Date Findings Submitted to:	
Dates of Inspection	Phase(s) Inspected	Study Director	Study Director Management
06-Feb-2008	Protocol Review	27-Mar-2008	27-Mar-2008
22-Feb-2008	Dosing	27-Mar-2008	27-Mar-2008
27-Mar-2008	Protocol Amendment Review	27-Mar-2008	27-Mar-2008
27-Mar-2008	Protocol Amendment Review	27-Mar-2008	27-Mar-2008
26-Mar-2008, 27-Mar-2008	Data Audit	27-Mar-2008	27-Mar-2008
27-Mar-2008	Draft Report Review	27-Mar-2008	27-Mar-2008
08-Oct-2008	Final Report Review	08-Oct-2008	08-Oct-2008

QA statement(s) provided by the following test site(s) have been reviewed:

Test Site(s)	Phase	QA Statement Location
Monsanto Company	Analytical Chemistry Report	Appendix 2

The final report has been reviewed to assure that it accurately describes the materials and methods, and that the reported results accurately reflect the raw data.

Jennifer D. McGue 08-Oct-2008

Jennifer D. McGue Date
Associate Quality Assurance Auditor
Charles River Laboratories
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4. INTELLECTUAL PROPERTY RIGHTS

5. NOTES TO REVIEWER

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6. RESPONSIBLE PERSONNEL

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7. SUMMARY

The purpose of this study was to evaluate the short-term toxicity of Delta 6 Desaturase and Delta 15 Desaturase following a single oral gavage administration to mice. The study design was as follows:

Experimental Design

Group No.	No. of Animals		Treatment	Analytically-Determined Dose Level (mg protein/kg body weight)
	Male	Female		
1	10	10	Bovine Serum Albumin (BSA)	44.0
2	10	10	Delta 6 Desaturase	4.66
3	10	10	Delta 15 Desaturase	37.3

The following variables and end points were evaluated in this study: clinical signs, body weights, body weight changes, food consumption, and gross necropsy findings.

Results:

No mortality occurred during the study, and no test article-related clinical findings were observed. There were no differences in body weights, body weight changes, or food consumption in Delta 6 Desaturase-treated or Delta 15 Desaturase-treated animals when compared to Bovine Serum Albumin-treated control animals. No test article-related gross necropsy findings were present.

Conclusion:

Delta 6 Desaturase induced neither mortality nor other adverse effects when administered to mice by single oral gavage at a dose of 4.66 mg/kg, nor was there mortality or other adverse effects induced by Delta 15 Desaturase when administered to mice by single oral gavage at a dose of 37.3 mg/kg. These doses are at least 100 times conservative estimates for potential human exposure to Delta 6 Desaturase and Delta 15 Desaturase.

8. INTRODUCTION

Monsanto has developed soybean, MON 87769, which produces stearidonic acid (SDA), an omega-3 fatty acid. Production of SDA in soybean seed was achieved through the introduction of genes encoding the production of Delta-6 (Δ6) and Delta-15 (Δ15) desaturases from *Primula juliae* and *Neurospora crassa*, respectively.

The purpose of this study was to evaluate the short-term toxicity of *Primula juliae* Delta 6 Desaturase (Delta 6 Desaturase, PjΔ6D) and *Neurospora crassa* Delta 15 Desaturase (Delta 15 Desaturase, NcΔ15D) isolated from immature MON 87769 soybean seeds following single oral gavage administrations to mice.

The protocol was signed by the Study Director on 06 February 2008 (GLP initiation date). The experimental start date was 14 February 2008 and the experimental completion date was 07 March 2008. The in-life phase of the study was initiated on 22 February 2008 and the in-life completion date was 07 March 2008.

9. MATERIALS AND METHODS

9.1. Test Materials

9.1.1. Test Articles

Test article 1 is defined as follows:

Identification	Delta 6 Desaturase (*Primula juliae* Delta 6 Desaturase from MON 87769 soybean seed)
ID Number	10001532

A dosing solution was prepared by the Sponsor from the above material and identified as follows:

Identification	Test Dosing Solution 1 (TDS1) – Diluted Delta 6 Desaturase
ID Number	10001532-D
Assigned Testing Facility ID	S08.004.EUF
Receipt Date	21-Feb-2008
Expiration Date	20-Feb-2009
Physical Description	Clear, colorless liquid
Storage Conditions	Frozen in a -70°C freezer
Supplier	Monsanto Research Center

Test article 2 is defined as follows

Identification	Delta 15 Desaturase (*Neurospora crassa* Delta 15 Desaturase from MON 87769 soybean seed)
ID Number	10001516

A dosing solution was prepared by the Sponsor from the above material and identified as follows:

Identification	Test Dosing Solution 2 (TDS2) – Diluted Delta 15 Desaturase
ID Number	10001516-D
Assigned Testing Facility ID	S08.005.EUF
Receipt Date	21-Feb-2008
Expiration Date	20-Feb-2009
Physical Description	Clear, slightly cloudy liquid
Storage Conditions	Frozen in a -70°C freezer
Supplier	Monsanto Research Center

9.1.2. Control Article

The control article is defined as follows:

Identification	Bovine Serum Albumin (BSA)
Manufacturer's ID Number	D0019305
Manufacturer	Calbiochem

A dosing solution was prepared by the Sponsor from the above material and identified as follows:

Identification	Control Dosing Solution (CDS) – Solubilized BSA
ID Number	D19305-C
Assigned Testing Facility ID	S08.003.EUF
Receipt Date	21-Feb-2008
Expiration Date	20-Feb-2009
Physical Description	Clear colorless liquid
Storage Conditions	Frozen in a -70°C freezer
Supplier	Monsanto Research Center

9.1.3. Test and Control Article Characterization

Certificates of Analysis for the test and control articles are presented in Appendix 1.

9.1.4. Reserve Sample

The Sponsor was responsible for maintaining retention samples of the test articles.

9.1.5. Inventory and Disposition

An inventory of the test materials supplied by the Sponsor was maintained. All unused dosing solutions were returned to the Sponsor following completion of the in-life phase.

9.1.6. Preparation of Dose Formulations

The dosing solutions were administered as received from the Sponsor. The dosing solutions were removed from the freezer, allowed to warm to room temperature, stirred at room temperature for at least 20 minutes prior to dosing, and stirred continuously during dosing.

9.1.7. Analysis of Dose Formulations

Dose formulation samples were collected for analysis as indicated in the following table:

Dose Formulation Samples for Analysis

Time Point	Concentration/Stability	Homogeneity
Day 0 (pre-dose)	Groups 1-3	Groups 2 and 3
Day 0 (post-dose)	Groups 1-3	N/A
Note: N/A = not applicable.		

9.1.7.1. Concentration and Stability

One 125-µL sample was collected from each dosing formulation on Day 0 (pre-dose and post-dose) for concentration and stability analyses.

9.1.7.2. Homogeneity

One 100-µL sample was collected from the top, middle, and bottom of the Group 2 and 3 dosing formulations on Day 0 (pre-dose) for homogeneity analysis. The CDS (Group 1) was determined to be a solution and was not sampled.

9.1.7.3. Analytical Sample Storage and Shipment

All samples and any unused dosing solutions were stored frozen (in a -70°C freezer) and shipped overnight on dry ice to the Sponsor for analysis. The Analytical Chemistry Report is presented in Appendix 2.

9.2. Test System

9.2.1. Receipt and Description

Fifty-eight male and 58 female CD-1 mice were received on 14 February 2008 from Charles River Laboratories, St. Constant, Quebec. Thirty-five animals per sex were assigned to this study. The animals were examined and weighed on the day following receipt, and were all allowed to acclimate to the laboratory environment for 8 days prior to the first day of dosing.

9.2.2. Justification of Test System/Route

The CD-1 mouse was chosen as the animal model for this study as it is a preferred rodent species for preclinical toxicity testing by regulatory agencies. The oral route of exposure was selected since this is a potential route of human exposure.

9.2.3. Housing

The animals were housed individually in suspended stainless steel cages during acclimation and while on study. Housing and care were as specified in the USDA Animal Welfare Act (9 CFR, Parts 1, 2, and 3) and as described in the *Guide for the Care and Use of Laboratory Animals*[1]. Targeted environmental conditions were as follows:

Temperature	64-79ºF (18-26ºC)
Humidity	50 ± 20%
Light Cycle	12-hour light/12-hour dark cycle
Air Changes	Ten or more air changes per hour with 100% fresh air

Actual room temperature and relative humidity were recorded a minimum of once daily and ranged from 69 to 71°F (21 to 22°C) and 43 to 51%, respectively.

9.2.4. Animal Identification

The animals were individually identified using metal ear tags and cage cards.

9.2.5. Food

PMI Nutrition International Certified Rodent Chow® #5002 was provided *ad libitum* throughout the study, except during fasting 2 to 3 hours prior to dosing. The lot number and expiration date of each batch of diet used during the study were recorded. The feed was analyzed by the supplier for nutritional components and environmental contaminants. Results of the dietary analyses were provided by the manufacturer and are maintained on file at the Testing Facility. Based on these results, there were no contaminants that would interfere with the conduct or interpretation of the study.

9.2.6. Water

Municipal tap water following treatment by reverse osmosis and ultraviolet irradiation was available *ad libitum* throughout the study. The water is periodically analyzed for total dissolved solids, hardness, microbiological content, and various potential environmental contaminants. Results of these analyses are maintained on file at the Testing Facility. Based on these results, there were no contaminants that would interfere with the conduct or interpretation of the study.

9.2.7. Veterinary Care

Veterinary care was available throughout the study and animals were examined by the Attending Veterinarian as warranted by clinical signs or other changes. All veterinary examinations and recommended therapeutic treatments were documented in the study records. No veterinary medicinal treatments were administered during the study.

9.2.8. Assignment to Study Groups

Prior to randomization procedures, 70 animals were weighed and examined in detail. Animals determined to be suitable as test subjects were then randomly assigned to groups by a stratified randomization scheme designed to achieve similar group mean body weights. Homogeneity of groups by weight was the criteria of acceptance of the randomization. At the time of randomization, the male animals selected for study use were approximately 8 weeks of age with body weights ranging from 28.5 to 33.8 grams and the female animals selected for study use were approximately 10 weeks of age with body weights ranging from 23.1 to 29.5 grams.

9.3. Experimental Design

The experimental design was as follows:

Experimental Design

Group No.	No. of Animals		Test Material [Control/Test Article]	Target Dose Level (mg protein/kg body weight)[a]	Dose Volume (mL dosing solution/kg body weight)
	Male	Female			
1	10	10	Control Dosing Solution (CDS) [Bovine Serum Albumin (BSA)]	35	33.3
2	10	10	Test Dosing Solution 1 (TDS1) [Delta 6 Desaturase]	1.75	33.3
3	10	10	Test Dosing Solution 2 (TDS2) [Delta 15 Desaturase]	35	33.3

[a]The target dose levels were selected to be at least 100 times conservative estimates of human exposure to the test proteins, Delta 6 Desaturase and Delta 15 Desaturase.

9.4. Administration of Test Materials

On Day 0, the animals chosen for use in the study were weighed and fasted for approximately 2 to 3 hours prior to dose administration. The test and control dosing solutions were administered once to the appropriate mice. The dose volume for each animal was based on the most recent non-fasted body weight measurement. The doses were given using a syringe with attached gavage cannula. The day of dosing was designated as Study Day 0.

10. EXPERIMENTAL PROCEDURES

10.1. Mortality/Moribundity Checks

General health/mortality and moribundity checks were performed twice daily, in the morning and afternoon.

10.2. Clinical Observations

Detailed clinical observations were performed two times on Day 0 (post dose) and once daily thereafter (Days 1-14). Each animal was removed from the cage and observed in detail as described in Appendix 3. A final detailed clinical observation was performed for each animal on the day of scheduled euthanasia.

10.3. Body Weights

Individual body weights were recorded on Day 0 prior to fasting, Day 0 prior to dosing, and on Days 7 and 14.

10.4. Food Consumption

Food consumption measurements were recorded on Days 0, 7, and 14.

10.4.1. Terminal Procedures

Terminal procedures are summarized in the following table:

Terminal Procedures

Group No.	No. of Male/Female Mice	Scheduled Euthanasia Day	Terminal Procedures	
			Gross Necropsy	Tissue Collection
1	10/10	14	x	x
2	10/10	14	x	x
3	10/10	14	x	x
Note: "x" = procedure conducted.				

10.4.1.1. Gross Necropsy

All animals were euthanized by carbon dioxide inhalation and subjected to a complete gross necropsy examination. The necropsy examination included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues.

10.4.1.2. Tissue Collection and Preservation

The lungs and GI tract of each animal were infused and the entire animal was retained in 10% neutral buffered formalin for possible future analysis.

10.5. Protocol Deviations

No protocol deviations were noted during the course of the study.

11. DATA ACQUISITION AND ANALYSIS

11.1. Electronic Data Acquisition/Systems

The in-life and gross pathology data were recorded on the Compaq Alpha DS10 Computer using the Toxicology Analysis System Customized, General Toxicology Module, Version 1.0.0 or higher. The temperature and humidity were recorded on a Systems 600 Apogee Insight System, Version 3.0 or higher. The following computer study numbers were used to collect data for the various study phases: EUF219, main phase data; and MS0805, acclimation data. The tables and appendices within this report display the applicable computer study number.

11.2. Statistical Analysis

Inferential statistical analyses were performed for the animals using the Compaq Alpha DS10 Computer. The following parameters and end points were analyzed: body weights, body weight changes, and food consumption

Each data set was subjected to a statistical decision tree. Data sets for each interval were initially analyzed for homogeneity of variance using Levene's test[2] followed by the Shapiro-Wilk test[3] for normality. A $p < 0.001$ level of significance was required for each test to reject the null hypothesis.

If neither Levene's test nor the Shapiro-Wilk test were significant, a single-factor parametric ANOVA[4] was applied, with animal grouping as the factor, using a $p < 0.05$ level of significance. If the parametric ANOVA was significant at $p < 0.05$, Dunnett's test was used to identify statistically significant differences between the control group and each test article-treated group using a minimum significance level of $p < 0.05$.

If either Levene's test and/or the Shapiro-Wilk test were significant, then the Kruskal-Wallis non-parametric ANOVA[5] was applied, with animal grouping as the factor, using a $p < 0.05$ level of significance. If the non-parametric Kruskal-Wallis ANOVA was significant at $p < 0.05$, Dunn's test[6] was used to identify statistically significant differences between the control group and each test article-treated group using a minimum significance level of $p < 0.05$.

12. MAINTENANCE OF RAW DATA, RECORDS, AND SPECIMENS

Following issuance of the Final Report, materials including but not limited to the protocol and protocol amendment(s), in-life records, pathology records, formulation records, correspondence related to the study, Final Report, micro-slides, specimens, wet tissues, slides, and blocks will be archived at the Testing Facility for a period of 3 years, after which the Sponsor will be contacted concerning continued storage or return of the materials.

Analytical and dosing preparation data will be archived by the Sponsor.

13. RESULTS

13.1. Dose Formulation Analysis

Appendix 2 (Analytical Chemistry Report)

The CDS (Group 1) was determined to be a stable solution with a BSA concentration of 1.32 mg/mL. TDS1 (Group 2) was determined to be a stable, homogeneous solution with a Delta 6 Desaturase protein concentration of 0.14 mg/mL. TDS2 (Group 3) was determined to be a stable, homogeneous solution with a Delta 15 Desaturase protein concentration of 1.12 mg/mL. The analytically-determined doses for Group 1 (BSA), Group 2 (Delta 6 Desaturase) and Group 3 (Delta 15 Desaturase) were 44.0 mg/kg body weight, 4.66 mg/kg body weight, and 37.3 mg/kg body weight, respectively.

13.2. Survival

Table 1 (Summary Data)
Appendix 4 (Individual Data)

No mortality occurred during the study. All animals survived until scheduled termination (Day 14).

13.3. Clinical Observations

Table 1 (Summary Data)
Appendix 4 (Individual Data)

No test article-related clinical signs occurred during the study. A male animal dosed with BSA (CDS) was observed with tremors and urine stain on the day of dosing. A single male animal dosed with Delta 6 Desaturase (TDS-1) was observed with a urine stain and unkempt appearance, and a single male dosed with Delta 15 Desaturase (TDS-2) was observed with unkempt appearance. There were no clinical signs in the females during the study. The clinical signs in the males were not considered meaningful as they were observed only in single animals and were observed in multiple dose groups.

13.4. Body Weights

Table 2 and Table 3 (Summary Data)
Appendix 5 and Appendix 6 (Individual Data)

There were no test article-related effects on body weight during the study. The mean weight gain was comparable throughout the groups. There were occurrences of slight decreases in body weight during the Day 0 to 7 interval for two males treated with Delta 6 Desaturase, two females treated with BSA, and a single female treated with Delta 6 Desaturase. Slight decreases in body weight during the Day 7 to 14 interval were noted in a single BSA-treated female, two females treated with Delta 6 Desaturase, and two females treated with Delta 15 Desaturase, with one Delta 15 Desaturase-treated female maintaining the same body weight from Day 7 to 14. With the relatively low number of animals showing the decrease, these changes were not considered

meaningful. There were no corresponding clinical signs or changes in food consumption to indicate health concerns.

13.5. Food Consumption

Table 4 (Summary Data)
Appendix 7 (Individual Data)

Food consumption was comparable throughout all groups. There were no test article-related effects on food consumption.

13.6. Gross Necropsy

Table 5 (Summary Data)
Appendix 8 (Individual Data)

One BSA-dosed male had a kidney cyst, one Delta 15 Deasaturase-dosed female was observed with depleted body fat, and one BSA-treated female and one Delta 15 Desaturase-treated female had periovarian cysts. Periovarian cysts are a common finding in this age and strain of mouse in this laboratory. Because of their isolated nature and the lack of a treatment-response, none of the gross necropsy findings are considered test article related.

14. CONCLUSION

Delta 6 Desaturase induced neither mortality nor other adverse effects when administered to mice by single oral gavage at a dose of 4.66 mg/kg, nor was there mortality or other adverse effects induced by Delta 15 Desaturase when administered to mice by single oral gavage at a dose of 37.3 mg/kg. These doses are at least 100 times conservative estimates for potential human exposure to Delta 6 Desaturase and Delta 15 Desaturase.

Jason W. Smedley 09OCT2008

Jason W. Smedley, B.S. Date
Study Director
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15. REPORT APPROVAL SIGNATURE

Mark A. Morse 09-OCT-2008

Mark A. Morse, Ph.D., DABT Date
Director of Research
Charles River Laboratories
Preclinical Services

16. REFERENCES

1. Guide for the care and use of laboratory animals. Washington, D.C.: National Academy Press. NRC (National Research Council); 1996.

2. Levene H. *Contributions to Probability and Statistics*. Stanford University Press; 1960.

3. Royston P, A remark on algorithm AS 181: the W-test for normality. *Applied Statistics*. 1995 44(4):547-551.

4. Gad SC and Weil CS. *Principles and Methods of Toxicology*. 3rd ed. New York, NY: Raven Press, Ltd.; 1994.

5. Siegel S. Nonparametric Statistics for the Behavioral Sciences; 1956.

6. Glantz SA. *Primer of Biostatistics*. 4th ed. The McGraw Hill Companies, Inc.; 1997.

Table 1
Summary of Survival and Clinical Observations

TABLE 1

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

MALES SUMMARY OF SURVIVAL AND CLINICAL OBSERVATIONS (TOTAL OBSERVATIONS/ANIMALS AFFECTED)

GROUP:	1	2	3
LEVEL MG/KG:	35	1.75	35
DOSE MATERIAL:	CDS	TDS1	TDS2
DAY 1 to 14			
NORMAL			
WITHIN NORMAL LIMITS	140/10	132/10	136/10
SCHEDULED EUTHANASIA	10/10	10/10	10/10
BODY			
URINE STAIN	0/ 0	3/ 1	0/ 0
UNKEMPT APPEARANCE	0/ 0	8/ 1	4/ 1

TABLE 1

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

FEMALES SUMMARY OF SURVIVAL AND CLINICAL OBSERVATIONS (TOTAL OBSERVATIONS/ANIMALS AFFECTED)

GROUP:	1	2	3
LEVEL MG/KG:	35	1.75	35
DOSE MATERIAL:	CDS	TDS1	TDS2
DAY 1 to 14			
NORMAL			
WITHIN NORMAL LIMITS	140/10	140/10	140/10
SCHEDULED EUTHANASIA	10/10	10/10	10/10

Table 2
Summary of Body Weight Data

TABLE 2

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

MALES SUMMARY OF BODY WEIGHT DATA (GRAMS)

	GROUP:		1	2	3
	LEVEL MG/KG:		35	1.75	35
	DOSE MATERIAL:		CDS	TDS1	TDS2
DAY 0 (NON-FASTED)		MEAN	31.0 d	31.1	30.4
		S.D.	1.52	1.69	2.84
		N	10	10	10
	% difference vs. control			0.3	-1.8
DAY 0 (FASTED)		MEAN	29.1 d	29.4	28.6
		S.D.	1.19	1.73	2.39
		N	10	10	10
	% difference vs. control			0.8	-2.0
DAY 7		MEAN	32.3 d	31.5	32.3
		S.D.	1.59	2.70	2.31
		N	10	10	10
	% difference vs. control			-2.4	-0.1
DAY 14		MEAN	34.1 d	34.2	34.2
		S.D.	1.43	2.46	2.68
		N	10	10	10
	% difference vs. control			0.3	0.2

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

TABLE 2

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

FEMALES SUMMARY OF BODY WEIGHT DATA (GRAMS)

GROUP:			1	2	3
LEVEL MG/KG:			35	1.75	35
DOSE MATERIAL:			CDS	TDS1	TDS2
DAY	0 (NON-FASTED)	MEAN	26.8 d	26.9	26.8
		S.D.	1.75	1.48	1.83
		N	10	10	10
	% difference vs. control			0.4	0.0
DAY	0 (FASTED)	MEAN	25.6 d	25.3	25.4
		S.D.	1.73	1.38	1.88
		N	10	10	10
	% difference vs. control			-1.2	-0.9
DAY	7	MEAN	27.4 d	27.1	27.9
		S.D.	1.44	1.24	1.43
		N	10	10	10
	% difference vs. control			-0.8	2.0
DAY	14	MEAN	28.3 d	28.2	29.3
		S.D.	1.83	2.03	1.63
		N	10	10	10
	% difference vs. control			-0.3	3.6

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

Table 3
Summary of Body Weight Changes

TABLE 3

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

MALES SUMMARY OF BODY WEIGHT CHANGES (GRAMS)

GROUP:			1	2	3
LEVEL MG/KG:			35	1.75	35
DOSE MATERIAL:			CDS	TDS1	TDS2
DAY	0 TO 7	MEAN	3.2 d	2.2	3.7
		S.D.	1.28	2.17	0.68
		N	10	10	10
DAY	7 TO 14	MEAN	1.8 d	2.6	1.8
		S.D.	0.89	1.10	0.69
		N	10	10	10

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

TABLE 3

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

FEMALES SUMMARY OF BODY WEIGHT CHANGES (GRAMS)

GROUP:			1	2	3
LEVEL MG/KG:			35	1.75	35
DOSE MATERIAL:			CDS	TDS1	TDS2
DAY	0 TO 7	MEAN	1.7 d	1.8	2.5
		S.D.	1.51	1.39	1.04
		N	10	10	10
DAY	7 TO 14	MEAN	1.0 d	1.1	1.4
		S.D.	1.06	1.23	1.27
		N	10	10	10

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

Table 4
Summary of Food Consumption Data

TABLE 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

MALES SUMMARY OF FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

		GROUP:	1	2	3
		LEVEL MG/KG:	35	1.75	35
		DOSE MATERIAL:	CDS	TDS1	TDS2
DAY	0 to 7	MEAN	6.4 d	6.1	6.3
		S.D.	0.50	1.00	1.02
		N	10	10	10
	% difference vs. control			-5.6	-2.4
DAY	7 to 14	MEAN	6.6 d	6.8	6.8
		S.D.	0.65	0.70	0.71
		N	10	9	10
	% difference vs. control			3.0	2.9

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

TABLE 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

FEMALES SUMMARY OF FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

	GROUP:		1	2	3
	LEVEL MG/KG:		35	1.75	35
	DOSE MATERIAL:		CDS	TDS1	TDS2
DAY	0 to 7	MEAN	5.1 d	5.4	5.7
		S.D.	0.93	0.76	0.59
		N	10	10	10
	% difference vs. control			6.3	10.7
DAY	7 to 14	MEAN	5.9 d	6.2	6.2
		S.D.	0.57	0.71	0.64
		N	10	10	10
	% difference vs. control			4.6	4.9

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

Table 5
Summary of Gross Necropsy Observations

TABLE 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS
SCHEDULED EUTHANASIA - DAY 14

GROUP:		1	2	3
LEVEL MG/KG:		35	1.75	35
DOSE MATERIAL:		CDS	TDS1	TDS2
MALES: TOTAL NUMBER EXAMINED		10	10	10
WITHIN NORMAL LIMITS		9	9	10
HAIRCOAT	N	0	1	0
WET MATTING	N	0	1	0
KIDNEY, CORTEX	N	1	0	0
CYST(S)	N	1	0	0

TABLE 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS
SCHEDULED EUTHANASIA - DAY 14

GROUP:		1	2	3
LEVEL MG/KG:		35	1.75	35
DOSE MATERIAL:		CDS	TDS1	TDS2
FEMALES: TOTAL NUMBER EXAMINED		10	10	10
WITHIN NORMAL LIMITS		9	10	8
CARCASS	N	0	0	1
BODY FAT DEPLETION	N	0	0	1
OVARY	N	1	0	1
PERIOVARIAN CYST(S)	N	1	0	1

Appendix 1
Certificates of Analysis

Analytical Protein Standard
Certificate of Analysis

MONSANTO *ANALYTICAL PROTEIN STANDARDS*

Sample Information:

Name of APS	**APS Lot Number**	**Expiration Date**
Seeds of Soy-produced *Primula juliae* Delta 6 Desaturase [MON 87769]	10001532	July 31, 2008

Common or Alias Name(s)	**Historical APS Lot Number**	**Storage Requirements (until use)**
PjD6D	—	–80 °C

Source	**Comment(s)**
Seeds of Soy MON 87769	None

Additional Background Information
None

Characteristic	Method	Assay Date	Result
Concentration	Amino Acid Composition	12 Feb 2008	0.52 mg/mL (total protein)
Purity	SDS-PAGE/Densitometry	14 Feb 2008	47%
Molecular weight	SDS-PAGE/Densitometry	14 Feb 2008	45.9 kDa
Identity	Immunoblot	29 Feb 2008	Confirmed – immuno reactive band observed
Identity	N-terminal sequence	27 Feb 2008	Confirmed – TKTIYITSSELEKHN
Identity	MALDI-TOF MS (Trypsinized)	28 Feb 2008	Confirmed sequence 42.2 % coverage of expected sequence

Buffer composition: 50 mM sodium acetate, pH 5.6, 1 mM $MgCl_2$, 0.1% Fos-choline 12, 0.5M NaCl and 10% glycerol.
Physical description: Clear solution
Short-term storage stability (29 days) was evaluated during the certification process. Based upon the criteria provided in Characterization Plan 10001532, no significant degradation was observed for samples stored at –80°C or -20º C. However, the relative molecular weight of the PjD6D protein had changed when stored at 4ºC.

Purity corrected concentration is 0.24 mg/mL (0.52 mg/mL × 0.47 ≈ 0.24 mg/mL)

Quality Assurance Specialist — Date: 04/29/08

Analytical Protein Standards Officer — Date: 4/29/08

Analytical Protein Standard
Certificate of Analysis

MONSANTO *ANALYTICAL PROTEIN STANDARDS*

Sample Information:

Name of APS	**APS Lot Number**	**Expiration Date**
Soy-produced *Neurospora crassa* Delta-15 Desaturase [MON 87769]	10001516	July 31, 2008
Common or Alias Name(s)	**Historical APS Lot Number**	**Storage Requirements (until use)**
NcD15D	—	–80 °C
Source		**Comment(s)**
Seeds of Soy MON 87769		None
Additional Background Information		
None		

Characteristic	Method	Assay Date	Result
Concentration	Amino Acid Composition	9 Feb 2008	0.62 mg/mL (total protein)
Purity	SDS-PAGE/Densitometry	14 Feb 2008	74%
Molecular weight	SDS-PAGE/Densitometry	14 Feb 2008	46.2 kDa
Identity	Immunoblot	05 Mar 2008	Confirmed – immuno-reactive band observed
Identity	N-terminal sequence	26 Feb 2008	Confirmed – AVTTRSHKAAAATEP
Identity	MALDI-TOF MS (Trypsinized)	27 Feb 2008	Confirmed sequence - 45.0 % coverage of expected sequence

Buffer composition: 50 mM sodium acetate, pH 5.6, 1 mM $MgCl_2$, 0.1% Fos-choline 12, 0.5M NaCl and 10% glycerol.
Physical description: Clear solution
Short-term storage stability (29 days) was evaluated during the certification process. Based upon the criteria provided in Characterization Plan 10001516, no significant degradation was observed for samples stored at –80°C or -20°C. However, the relative molecular weight of the NcD15D protein had changed when stored at 4°C.

Purity corrected concentration is 0.24 mg/mL (0.62 mg/mL × 0.74 ≈ 0.46mg/mL)

Quality Assurance Specialist — Date: 04/29/08

Analytical Protein Standards Officer — Date: 4/29/08

Appendix 2
Analytical Chemistry Report

Analytical Sub-Report Title

Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with Soybean MON 87769-Produced *Primula juliae* Delta-6 Desaturase and *Neurospora crassa* Delta-15 Desaturase Proteins

Authors

Jian G. Dong, Ph.D., Thomas C. Lee, Ph.D., Elena A. Rice, Ph.D.

Analytical Sub-Report Completed On

September 9, 2008

Performing Laboratory

Monsanto Company
800 North Lindbergh Boulevard
Saint Louis, Missouri 63167

Laboratory Project ID

MSL #: 0021314
Charles River Study #: EUF00219
Monsanto Study #: CRO-2007-324

Monsanto Company **Monsanto Study #: CRO-2007-324**
MSL-0021314
Regulatory Product Characterization Center **Page 2 of 29**

Statement of Compliance

This study meets the US EPA Good Laboratory Practices specified in 40 CFR Part 160 (EPA).

Submitter: _____ Date: _____

Sponsor Representative: _____ Date: 9-9-08
Terry A. Kaempfe, B.S.

Principal Investigator: _____ Date: 09-09-08
Jian G. Dong, Ph.D.

Quality Assurance Statement

Analytical Sub-Report Title: Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with Soybean MON 87769-Produced *Primula juliae* Delta-6 Desaturase and *Neurospora crassa* Delta-15 Desaturase Proteins

Charles River Study No.: EUF00219
Monsanto Study No.: CRO-2007-324

Reviews conducted by the Quality Assurance Unit confirm that the final analytical sub-report accurately describes the methods and standard operating procedures followed and accurately reflect the raw data of the study.

Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on the study reported herein.

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
03/14/08-03/31/08	Amino Acid Analysis of Dosing Samples	04/15/2008	04/15/2008
07/16/2008	Raw Data Audit	07/16/2008	07/16/2008
08/18/2008; 08/19/2008	Draft Report Audit	09/09/2008	09/09/2008

Quality Assurance Unit
Monsanto Regulatory, Monsanto Company

09/09/2008
Date

Analytical Sub-Report Certification

This report is an accurate and complete representation of the study/project activities.

Principal Investigator: Jian G. Dong, Ph.D. Date: 09-09-08

Study Information

Charles River/Monsanto Study Number:	EUF00219/CRO-2007-324
Analytical Sub-Report Title:	Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with Soybean MON 87769-Produced *Primula juliae* Delta-6 Desaturase and *Neurospora crassa* Delta-15 Desaturase Proteins
Facilities:	Monsanto Company 800 North Lindbergh Boulevard Saint Louis, Missouri 63167, USA
Study Director:	Jason W. Smedley, B.S.
Principal Investigator:	Jian G. Dong, Ph.D.
Contributors:	Bin Chen, John Finnessy, Tallis Brown, Chris Dalton, and Richard Thoma
Study Specific Work Procedure Initiation Date:	February 15, 2008
Analytical Sub-Report Completion Date:	September 09, 2008
Records Retention:	All Study-Specific Work Procedure raw data, Study-Specific Work Procedure plan and amendments, final sub-report and facility records were retained at Monsanto, St. Louis.
Disposition of Remaining Dosing Solutions:	Dosing samples were returned to Monsanto and disposed at the close of the study.

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Abbreviations[1]

AAA	Amino acid analysis
APS	Analytical Protein Standard
BSA	Bovine serum albumin
BW	Body weight
CDS	Control Dosing Solution
CFR	Code of Federal Regulations
COA	Certificate of analysis
CV	Coefficient of variance
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
MW	Molecular weight
MSL	Monsanto Scientific Literature
NcΔ15D	*Neurospora crassa* delta-15 desaturase
NIST	National Institute of Standards and Technology
PjΔ6D	*Primula juliae* delta-6 desaturase
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard operating procedure
TDS	Test dosing solution
US EPA	United States Environmental Protection Agency

[1] Standard abbreviations, e.g. units of measure, concentration, mass, time., are used without definition according to the format described in "Instructions to Authors" in The Journal of Biological Chemistry.

1.0 Summary

This analytical sub-report describes the formulation and subsequent analyses of the *Primula juliae* delta-6 desaturase (PjΔ6D), *Neurospora crassa* delta-15 desaturase (NcΔ15D), and the bovine serum albumin (BSA) proteins in the test dosing solutions (TDS1, TDS2) and control dosing solution (CDS), respectively. These dosing solutions were used in a mouse acute oral toxicity study performed at Charles River Laboratories, Inc. (Spencerville, OH).

The PjΔ6D, NcΔ15D and BSA proteins were formulated separately in the vehicle buffer containing 50 mM sodium phosphate, pH 7.4, 0.25 M NaCl, 0.5% (w/v) Triton X-100 (reduced), 0.02% (w/v) Fos-choline 12, and 10% glycerol. Samples of TDS1, TDS2, and CDS were collected prior to the initiation (pre-dose) and after the completion (post-dose) of the dosing to assess the concentration and stability of the dosing solutions administered to mice. The stability was assessed by determining the purity of the PjΔ6D, NcΔ15D and BSA proteins in the pre- and post-dose solution samples. The purity was determined using densitometric analyses of the samples separated on SDS-polyacrylamide (SDS-PAGE) gels followed by colloidal Brilliant Blue G stain. Total protein concentration of the pre- and post-dose TDS1, TDS2, and CDS samples was determined using amino acid analysis. The homogeneity of the TDS1 and TDS2 was assessed by comparing the protein concentrations of TDS aliquots taken from the top, middle, and bottom portions of their respective test dosing solutions prior to dosing.

The purity of the PjΔ6D protein was 48.9% and 49.7% in the pre- and post-dose TDS1 samples, respectively. The purity of the NcΔ15D protein was 71.3% and 74.4% in the pre- and post-dose TDS2 samples, respectively. The purity of the BSA protein in pre- and post-dose CDS samples was 80.6% and 79.8%. The TDS1, TDS2 and CDS samples were considered to be stable because they satisfied the preset criteria of less than 10% difference in purity between the pre- and post-dosing samples.

The TDS1 and TDS2 were determined to be homogenous based on the total protein concentration of the samples taken from the top, middle, and bottom portions of the containers containing dosing solutions.

The concentrations of the PjΔ6D protein in TDS1, the NcΔ15D protein in TDS2 and the BSA protein in the CDS were calculated as the average of protein concentration values determined for pre- and post-dose samples; corrected for purity. The concentrations of TDS1, TDS2 and the CDS were determined to be 0.14 mg/ml, 1.12 mg/ml, and 1.32 mg/ml, respectively. The dose levels were calculated using the average, purity-corrected concentration of each protein in the corresponding dosing solution. The dose level of PjΔ6D in TDS1 was 4.66 mg/kg body weight (BW). The dose level of the NcΔ15D in TDS2 was 37.3 mg/kg BW. The dose level of the BSA in the CDS was 44.0 mg/kg BW.

These data establish that the target dose levels were achieved and demonstrate the stability of the test and control dosing solutions used in the mouse oral acute toxicity study.

2.0 Introduction

An acute oral toxicity study was performed on mice as part of the safety assessment of the *Primula juliae* delta-6 desaturase (PjΔ6D) and *Neurospora crassa* delta-15 desaturase (NcΔ15D) proteins. This sub-report describes the formulation and analyses of the test and control dose solutions used in the mouse acute oral toxicity study. Analyses included total protein concentration and purity of the dose solutions (TDS1, TDS2 and CDS) both before and after dosing of mice. Additionally, the homogeneity of the two test dosing solutions was assessed. These procedures were performed to evaluate the administered dose concentrations and to assess if any changes in the test articles or control article occurred during the performance of the acute oral toxicity study.

3.0 Purpose

The purpose of this analytical sub-report is to describe the procedure for the preparation, formulation and analyses of the test and control dose solutions used in the mouse acute oral toxicity study performed at Charles River Laboratories Inc. (Charles River study number EUF00081; Spencerville, OH

4.0 Materials

4.1 Test Articles

The test articles were the MON 87769-produced PjΔ6D (Orion lot #: 10001532) and NcΔ15D proteins (Orion lot #: 10001516). Both proteins were isolated from immature soybean seeds of soybean MON 87769. The identity, concentration, purity, and stability of PjΔ6D and NcΔ15D proteins have been determined under characterization plans 10001532 and 10001516, respectively, and are described in the Certificates of Analysis (COA). Copies of the COAs are archived with this study file. PjΔ6D protein (Orion Lot 10001532) identified as Test Article 1, has a total protein concentration of 0.52 mg/ml with a purity of 47% while NcΔ15D protein (Orion Lot 10001516) identified as Test Article 2, has a total protein concentration of 0.62 mg/ml with a purity of 74%. Prior to use, these test articles were stored in a -80ºC freezer in a buffer solution containing 50 mM sodium acetate, pH 5.6, 500 mM NaCl, 1.0 mM $MgCl_2$, 10% glycerol, and 0.1% (w/v) Fos-choline 12.

4.2 Control Article

The control article was bovine serum albumin (BSA) protein (lot D00019305) purchased from Calbiochem (Cat. No. 126609). The vendor's COA is archived with this study file. According to the vendor's characterization, the protein has a purity of 98%. The relative amount of protein in the solid BSA powder was estimated by amino acid analysis as 83% (wt/wt; Monsanto Study CRO-2007-182). A copy of the COA is archived with this study file. In this study, a purity value of 81% (83% *x* 98% = 81%, wt/wt) was used in determining the BSA amount for CDS formulation.

4.3 Assay Controls

Protein molecular weight markers (Bio-Rad Broad Range, Hercules, CA) were used to calibrate SDS-polyacrylamide gels. Amino acid calibration standards (National

Institutes of Standards and Technology - NIST, Gaithersburg, MD) were used to calibrate the amino acid analyzer and NIST BSA was used as a hydrolysis control.

5.0 Dose Preparation

5.1 Calculation of the Protein Concentrations Required to Formulate Test Article Dosing Solutions and the Test Control Dosing Solution

The desired protein dose levels for PjΔ6D and NcΔ15D were estimated based on their expression levels in the article of commerce, the seeds. To ensure a minimum of 100 times margin of exposure, the desired dose level for PjΔ6D was set at 2.2 mg/kg BW while the desired dose level for NcΔ15D was set at 37.5 mg/kg BW. Theoretical protein concentrations required to formulate TDS1, TDS2 and CDS were calculated based on the assumptions described below.

5.1.1 Calculation of PjΔ6D Concentration in TDS1

a) The desired dose level for PjΔ6D in TDS1 is at least 2.2 mg/kg BW.
b) The average mouse body weight (BW) is 0.030 kg.
c) Dose solutions are administered at a final dosing volume of 33.3 ml/kg BW (~1 ml per mouse) as a single dose.

$$2.2\ mg/kgBW \quad x \quad 0.030\ kgBW/mouse \quad = \quad 0.066\ mg/mouse$$

Therefore, at least 0.066 mg of PjΔ6D is required in ~ 1 ml aliquots.
d) 20 mice will be dosed.
e) The minimal total volume of dose solution required is 20 ml:

$$20\ mice\ x\ 1\ dose\ x\ 1\ ml \quad = \quad 20\ ml$$

The estimated purity of the PjΔ6D preparation is approximately 47%. Therefore, the TDS1 solution should contain approximately 0.14 mg/ml of total protein:

5.1.2 Calculation of PjΔ15D Concentration in TDS2

a) The desired dose level for NcΔ15D in TDS2 is at least 37.5 mg/kg BW.
b) The average mouse body weight (BW) is 0.030 kg.
c) Dose solutions are administered at a final dosing volume of 33.3 ml/kg BW (~1 ml per mouse) as a single dose.

$$37.5\ mg/kgBW \quad x \quad 0.030\ kgBW/mouse \quad = \quad 1.12\ mg/mouse$$

Therefore, at least 1.12 mg of NcΔ15D required in ~ 1 ml aliquots.
d) 20 mice will be dosed.
e) The minimal total volume of dose solution required is 20 ml:

$$20\ mice\ x\ 1\ dose\ x\ 1\ ml \quad = \quad 20\ ml$$

The estimated purity of the NcΔ15D preparation is approximately 74%. Therefore, the TDS2 solution should contain approximately 1.5 mg/ml of total protein:

$$\frac{1.12\ mg/ml}{0.74} \cong 1.5\ mg/ml$$

5.1.3 Calculation of BSA Concentration

BSA was used to formulate the CDS at a purity-corrected concentration (1.12 mg/ml ± 10%) similar to that of TDS2 since the calculated required concentration of TDS2 is higher than that of TDS1.

The purity of the total protein in the solid BSA powder is approximately 83% and the purity of BSA in the total protein was about 98%. In this study, a purity value of 81% (83% *x* 98% = 81%) was used in determining the BSA amount required for the formulation. Therefore, the total protein concentration of CDS solution should be approximately 1.38 mg/ml:

$$\frac{1.12\ mg/ml}{0.83x0.98} \cong 1.38\ mg/ml$$

The experimentally obtained dose levels were determined after administration to mice by assessing the concentration and purity for the PjΔ6D, NcΔ15D, and BSA proteins in TDS1, TDS2 and the CDS, respectively.

5.2 Formulation of the Test Articles in the Test Dosing Solutions (TDS1 and TDS2)

For the preparation of the test dosing solutions (TDS1 and TDS2), the test article proteins were exchanged by column chromatography into a vehicle buffer containing 50 mM sodium phosphate, pH 7.4 , 250 mM NaCl, 0.02% (w/v) Fos-choline 12, 0.5% (v/v) Triton X-100 (reduced form), and 10% glycerol. For CDS preparation, the commercially available BSA was dissolved directly in the vehicle buffer.

5.2.1 Formulation of PjΔ6D Protein in the TDS1

To prepare TDS1, the PjΔ6D test article in 50 mM sodium acetate, pH 5.6, 0.1% (w/v) Fos-choline 12, and 10% glycerol was diluted 6-fold with 50 mM sodium acetate, pH 5.6, 0.1% (w/v) Fos-choline 12, and 10% (v/v) glycerol. The diluted solution was then loaded onto an 1.0-ml SP-Sepharose column (GE Healthcare, Cat. 17-5054-01) followed by washing with 1 bed volume of 10 mM sodium acetate, pH 5.6, 0.04% Fos-choline 12, and 10% glycerol to reduce the concentrations of Fos-choline 12 and sodium acetate. The protein was eluted from the column with 50 mM sodium phosphate, pH 8.5, 500 mM NaCl, 0.04% Fos-choline 12, and 10% glycerol. Peak fractions were collected and combined. The PjΔ6D-fractions (~15 mL) was diluted with an equal volume of 50 mM sodium phosphate, pH 7.4, 1.0% Triton (reduced), and 10% glycerol to a final buffer composition of 0.02% (w/v) Fos-choline 12, 0.5% (v/v) Triton X-100 (reduced), 10% glycerol, and 250 mM NaCl. The pH of the eluted fractions was

adjusted to pH 7.4 with diluted HCl or NaOH. The final formulated protein solution (~30 mL) is identified as TDS1. The final buffer composition of TDS1 consisted of 50 mM sodium phosphate, pH 7.4, 0.25 M NaCl, 0.02% (w/v) Fos-choline 12, 0.5% (w/v) Triton X-100 (reduced) and 10% glycerol.

5.2.2 Formulation of NcΔ15D in the TDS2

To prepare TDS2, the NcΔ15D test article solution was diluted 6-fold with 50 mM sodium acetate, pH 5.6, 0.1% (w/v) Fos-choline 12, and 10% glycerol. The diluted solution was loaded onto an 1.0-ml SP-Sepharose column (GE Healthcare, Cat. 17-5054-01) followed by washing with 1 bed volume of 10 mM sodium acetate, pH 5.6, 0.04% Fos-choline 12, 0.5% (w/v) Triton X-100 and 10% glycerol to reduce the concentrations of Fos-choline 12 and sodium acetate. The protein was eluted off the column with 50 mM sodium phosphate, pH 8.5, 1.0 M NaCl, 0.02% (w/v) Fos-choline 12, 0.5% (w/v) Triton X-100 (reduced form) and 10% glycerol. Peak fractions were collected and combined (~24 ml). The pH of the protein preparation was adjusted to pH 7.4 with diluted HCl or NaOH. The NcΔ15D-containing protein solution was diluted 2-fold with 50 mM sodium phosphate, pH 7.4, and 10% glycerol to reduce the concentration of NaCl to ~250 mM. The volume of the diluted solution was reduced by Amicon (Amicon Ultra-15, 10,000 MWCO cat. # UFC901024) filtration to a final volume of ~24 mL. The final formulated NcΔ15D is identified as TDS2. The final buffer composition of TDS2 consisted of 50 mM sodium phosphate, pH 7.4, 0.25 M NaCl, 0.02% (w/v) Fos-choline 12, 0.5% Triton X-100 (reduced) and 10% glycerol.

Following formulation, the TDS1 and TDS2 (~25 ml each) were each transferred to a separate wide mouth container, equipped with a Teflon stir bar. Aliquots of TDS1 and TDS2 were taken for suitability analyses. The dosing solutions were stored in a -80°C freezer until they were shipped on dry ice to the Testing Facility.

Concentration and SDS-PAGE analyses of TDS1 and TDS2 were conducted using the sample aliquots taken from the formulated doses. TDS1 and TDS2 were evaluated for their suitability for the study using the following criteria: (1) the dose solution should pass through an 18 or 20-guage needle; and (2) a similar pattern of stained protein bands relative to the test articles should be observed by SDS-PAGE analysis.

5.2.3 Formulation of BSA in the Control Dosing Solution (CDS)

BSA powder (56.0 mg, Calbiochem Cat. No. 126609, lot D00019305) was weighed and placed in a 50 ml Falcon tube and brought to ~30 ml total volume in the vehicle buffer containing 50 mM sodium phosphate, pH 7.4 , 250 mM NaCl, 0.02% (w/v) Fos-choline 12, 0.5% (w/v) Triton X-100 (reduced form), and 10% glycerol. The CDS (~30 ml) was transferred to a wide mouth container, equipped with a Teflon stir bar and stored in a -80°C freezer until it was shipped on dry ice to the Testing Facility.

5.3 Samples Received from the Testing Facility

The following samples of the TDS1, TDS2 and CDS were received from the Testing Facility (samples were returned on dry ice) for analysis. All samples were stored in a –80°C freezer prior to analysis.

Time Point	Dose Solution Identification	Analysis	Sample Volume (µl)	Sample Identification
Pre-dose	CDS	Concentration/Stability	~ 125	Pre-CDS
	TDS1	Concentration/Stability	~ 125	Pre-TDS1
	TDS1	Homogeneity –Bottom	~ 100	B-TDS1
	TDS1	Homogeneity – Middle	~ 100	M-TDS1
	TDS1	Homogeneity – Top	~ 100	T-TDS1
Pre-dose	TDS2	Concentration/Stability	~ 125	Pre-TDS2
	TDS2	Homogeneity –Bottom	~ 100	B-TDS2
	TDS2	Homogeneity – Middle	~ 100	M-TDS2
	TDS2	Homogeneity – Top	~ 100	T-TDS2
Post-dose	CDS	Concentration/Stability	~ 125	Post-CDS
	TDS1	Concentration/Stability	~ 125	Post-TDS1
	TDS2	Concentration/Stability	~ 125	Post-TDS2

6.0 Methods

A list of applicable SOPs for the methods described below is shown in Appendix 1.

6.1 Amino Acid Analysis

The concentrations of TDS1, TDS2 and CDS samples were determined by amino acid analysis (AAA) using AccQ-Tag® derivatization (Waters Corporation, Milford, MA), which allows for high sensitivity fluorescent detection of amino acids. The same analytical procedures were used for both the suitability and confirmation analysis samples. In order to avoid the interference from buffer components during protein hydrolysis, protein samples were precipitated using ethanol. The sample preparation was as follows: in a hydrolysis tube (~300 µl), approximately 1µg of protein samples were mixed with 200 µl of chilled 95% ethanol. After incubation overnight at -20ºC, samples were centrifuged at 12,000 rpm in a microcentrifuge for 30 min at 2-8°C. The supernatant was removed and discarded. Precipitates were then washed sequentially with 100 µl of both chilled acetone and water. Along with replicates of the test samples, a hydrolysis blank, 4 dilutions of a calibration standard

(NIST), and a NIST BSA control were also analyzed. The internal calibrant, α-aminobutyric acid, was included in all non-blank samples. All samples were evaporated to dryness in hydrolysis tubes using a Speed-Vac concentrator. 500 µl of hydrolysis solution (6N HCl/1% phenol) was added and the tubes were transferred to a vacuum chamber. Samples were hydrolyzed for 90 min at 150±2°C under vacuum. After cooling, the vacuum was released and the hydrolysates were evaporated to dryness using a Speed-Vac concentrator and reconstituted in 20 µl of 20 mM HCl. The tubes were vortexed to resuspend the samples. A 60 µl aliquot of AccQ-Fluor Borate Buffer and a 20 µl aliquot of AccQ-Fluor reagent were added sequentially to each vial with vortexing after each addition. The samples were transferred individually to autosampler vials, capped, and heated to 55 °C for 10 min. Samples were analyzed using a 2695 Separation Module (Waters Corp.) equipped with a reverse-phase C-18 column for detection of AccQ-Tag derivatized amino acids. Chromatographic data were collected using Atlas software (Thermo Electron Corp.). Dosing solutions were considered to be stable for the duration of the dosing period if a ≤15% change in protein concentration between the pre- and post-dose samples was observed.

6.2 SDS-PAGE and Purity Analysis

For the evaluation of TDS suitability, the protein banding patterns of the formulated dosing solutions were compared to the banding patterns of their corresponding test article proteins, PjΔ6D (Orion # 10001532) and NcΔ15D (Orion # 10001532). Due to the lower sensitivity of PjΔ6D to the staining dye, slightly higher amount of total protein of PjΔ6D preparations were loaded on SDS-PAGE gels. Aliquots of 2, 3 and 4 µg total protein of both the PjΔ6D test article protein and the formulated TDS1were loaded simultaneously on a pre-cast tris-glycine 4-20% polyacrylamide gradient mini-gels (Invitrogen, Carlsbad, CA). Similarly, aliquots of 1, 2 and 3 µg total protein of both the NcΔ15D test article protein and the formulated TDS2 were loaded simultaneously on a pre-cast tris-glycine 4-20% polyacrylamide gradient mini-gels. SDS-PAGE gels were run under reducing conditions at 50 V for 25 min followed by 160 V for 70 min. The gels were stained with Colloidal Brilliant Blue G (Sigma, Chemical Co., St. Louis, MO), and de-stained (Destain A) for 30-45 sec. in a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid followed by further de-staining (Destain B) in a solution of 25% methanol for 6-10 h. The protein profiles of the test articles and their corresponding formulated TDSs were visually compared.

Upon completion of the dose administration to mice, samples of TDS1, TDS2 and CDS were analyzed by SDS-PAGE. TDS1 was loaded at 2, 3 and 4 µg total protein per lane on SDS-PAGE; whereas TDS2 and CDS were loaded at 1, 2 and 3 µg total protein per lane. SDS-PAGE was performed using pre-cast tris-glycine 4-20% polyacrylamide gradient mini-gels (Invitrogen, Carlsbad, CA) under reducing conditions. Broad range molecular weight markers (Bio-Rad, Cat. No. 161-0317, Hercules, CA) were used to estimate molecular weights. All dilutions were made in a buffer (50 mM sodium phosphate, pH 7.4, 0.25 M NaCl, 0.02% (w/v) Fos-choline 12, 0.5% Triton and 10% glycerol) and then applied to a 10-well mini-gel.

Electrophoresis was performed at constant voltage at 50 V for 25 min followed by 160 V for 70 min. Proteins were fixed with 40% (v/v) methanol, 7 % (v/v) acetic acid, stained (16-20 h) by gentle shaking with Colloidal Brilliant Blue G (Sigma, Chemical Co., St. Louis, MO), and de-stained (Destain A) for 30-45 sec. in a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid followed by further de-staining (Destain B) in a solution of 25% methanol for 6-10 h. Purity and molecular weight were evaluated using a Bio-Rad GS-800 calibrated densitometer equipped with Quantity One® software (version 4.4.0 build 036). Molecular weight was evaluated for positional reference only. The purity was calculated as the mean of all three lane loads. Dosing solutions were considered to be stable for the duration of the dosing period if a ≤10% change in Desaturase or BSA protein purity between the pre- and post-dose samples was observed.

6.3 Statistical Methods

SD and % CV values for TDS and CDS concentration and purity data were calculated using Microsoft® Excel 2000 software (version 9.0.7616 SP-3).

7.0 Control of Bias and Quality Measures

Appropriate sets of dosing solution samples were analyzed concurrently on SDS-PAGE gels to eliminate any run-to-run variability.

8.0 Changes to the Study-Specific Work Procedure

There were no amendments made to the Study-Specific Work Procedure. There were two SOP deviations:

1. The preparation of the AccQ-Fluor reagent was not documented as required by the Reagent and Solution Documentation SOP AG-PO-1163-02. There is no impact to the analysis due to the deviation as the preparation of the AccQ-Flour reagent was made and stored in accordance to SOP BR-ME-1139-01.
2. One of the suitability criteria stated in SOP BR-ME-1139-01, the chromatographic suitability criterion (SOP BR-ME-1139-01) during AAA analysis regarding run to run peak areas for Asp and Phe was exceeded. The integrity of the data is orthogonally verified by the other suitability criteria, notably the calibration and hydrolysis suitability criteria. There was no adverse impact on the result of this analysis due to this deviation.

9.0 Data Rejected

There were no data rejected during the course of study.

10.0 Results and Discussion

10.1 Dosing Solution Formulation and Suitability Assessment

The buffer of the test articles (Orion lot #s 10001532 and 10001516) contains 0.1% (w/v) Fos-choline 12, a detergent which is necessary to keep the proteins of interest in solution. However, Fos-choline 12 could be a toxicity concern in mice. For dose preparation, the concentration of the PjΔ6D and NcΔ15D proteins needed to be

adjusted without simultaneous increase in the detergent concentration. To achieve this, we employed cation exchange chromatography to perform protein concentration and detergent exchange by partially replacing Fos-choline 12 with 0.5% Triton X-100, a non-ionic detergent which mice can tolerate. Following the buffer exchange, both proteins remained soluble at the target protein concentrations in the presence of 0.5% (w/v) Triton X-100 (reduced) and 0.02% (w/v) Fos-choline 12.

Dosing solutions containing the PjΔ6D, NcΔ15D and BSA proteins were prepared based on the calculations and assumptions described in Section 5.1. Several criteria were set to test the suitability of the TDSs for use in the study. Both TDS1 and TDS2 must pass through an 18-gauge needle so that the samples are readily administered through the oral (gavage) rout to mice. The total protein concentrations of TDS1 and TDS2 must be equal to or higher than 0.14 mg/ml for TDS1 and 1.54 mg/ml for TDS2 in order to achieve the desired dose levels. Similar protein banding patterns must be observed between the TDS samples and their corresponding test articles on the stained SDS-PAGE gels. Both TDS1 and TDS2 passed all three criteria, thus shown to be suitable for use in this study.

10.2 Evaluation of TDS Homogeneity

TDS homogeneity was determined by evaluating the protein concentration of samples taken from the top, middle, and bottom sections of the TDS solution container. The CV of the average concentration of the three samples determined by AAA was 4.5% and 3.8% for the TDS1 and TDS2, respectively (Tables 1 and 2). These values were below the pre-set acceptance criterion for homogeneity of ≤ 15% and, therefore, the TDS1 and TDS2 were considered to be a homogeneous solutions.

10.3 Protein Concentration of TDS1, TDS2 and the CDS

Total protein concentration was determined using amino acid analysis on samples taken before and after administration of doses (Tables 3, 4 and 5). The total protein concentrations for the pre- and post-dose TDS1 samples were determined to be 0.258 mg/ml and 0.294 mg/ml, respectively (Table 3). The average concentration of the TDS1 samples was calculated as 0.28 mg/ml.

The total protein concentrations for the pre- and post-dose TDS2 samples were determined to be 1.543 mg/ml and 1.528 mg/ml, respectively (Table 4). The average concentration of the TDS2 samples was calculated as 1.54 mg/ml.

The total protein concentrations for the pre- and post-dose CDS samples were determined as 1.641 mg/ml and 1.645 mg/ml, respectively (Table 5). The average concentration of the CDS samples was calculated as 1.64 mg/ml.

10.4 Purity of the PjΔ6D, NcΔ15D and BSA Proteins in the TDS1, TDS2 and CDS

To assess the stability of the PjΔ6D, NcΔ15D and BSA proteins in the respective TDS1, TDS2 and CDS solutions; the protein purity of pre- and post-dose samples was determined using densitometric analysis of Colloidal Brilliant Blue G stained

SDS-polyacrylamide gels (Figures 1, 2 and 3). Purity data for the PjΔ6D, NcΔ15D and BSA proteins are summarized in Table 6.

The average percent purity of PjΔ6D protein in the pre- and post-dose TDS1 samples was 48.9% and 49.7%, respectively. The percent change in purity was 1.6% (Table 6), which was within the preset stability criteria of <10% variation. Therefore, TDS1 was considered to be stable for the duration of the dosing period.

The average percent purity of NcΔ15D protein in the pre- and post-dose TDS2 samples was 71.3% and 74.4%, respectively. The percent change in purity was 4.3% (Table 6), which was within the preset stability criteria of <10% variation. Therefore, TDS2 was considered to be stable for the duration of the dosing period.

The average purity of BSA was estimated to be 80.6% and 79.8% in pre- and post-dose CDS samples, respectively. The percent change in purity was 1.0% (Table 6), which was within the preset stability criteria of <10% variation. Therefore, the CDS was considered to be stable for the duration of the dosing period.

10.5 Calculation of the TDS1, TDS2 and CDS Dose Levels

The final dose levels were calculated using concentration and purity values determined for the TDS1, TDS2 and CDS (Table 3, Table 4 and Table 5). Because the TDSs were determined to be homogeneous suspensions, the administered test article dose levels were calculated from the average concentration of the pre- and post- dose values. The calculations are shown below and the results are summarized in Table 7.

The dose level for the TDS1 was determined as $4.66 \frac{mg}{kg\ BW}$:

a) Concentration corrected for purity: $0.276 \frac{mg}{ml} \times 0.493 = 0.14 \frac{mg}{ml}$

b) Protein level per dose: $0.14 \frac{mg}{ml} \times 33.3 \frac{ml}{kgBW} = 4.66 \frac{mg}{kgBW}$

The dose level for the TDS2 was determined as $37.3 \frac{mg}{kg\ BW}$:

a) Concentration corrected for purity: $1.54 \frac{mg}{ml} \times 0.728 = 1.12 \frac{mg}{ml}$

b) Protein level per dose: $1.12 \frac{mg}{ml} \times 33.3 \frac{ml}{kgBW} = 37.3 \frac{mg}{kgBW}$

The dose level for the CDS was determined as 44.0 $\frac{mg}{kg\ BW}$:

a) Concentration corrected for purity: $1.64\ \frac{mg}{ml} \times 0.802 = 1.32\ \frac{mg}{ml}$

b) Protein level per dose: $1.32\ \frac{mg}{ml} \times 33.3\ \frac{ml}{kgBW} = 44.0\ \frac{mg}{kgBW}$

11.0 Conclusions

The analytical tests performed for the TDS samples established that stable, homogenous formulations of the PjΔ6D and NcΔ15D proteins in the TDS1 and TDS2, respectively, were achieved. No change in the purity of the PjΔ6D and NcΔ15D proteins was observed prior to and after the administration of the TDSs to mice, indicating that the TDSs were stable throughout the dosing. No change in the BSA protein purity was observed prior to or after the administration of the CDS to mice, indicating that the CDS was stable throughout the dosing period.

The concentration of the PjΔ6D, NcΔ15D, and BSA proteins in the TDS1, TDS2, and CDS, respectively, were calculated based upon average total protein concentration and percent purity. The experimentally confirmed dosing level of the PjΔ6D protein in the TDS1 was 4.66 mg/kg mouse BW. The experimentally confirmed dosing level of the NcΔ15D protein in the TDS2 was 37 mg/kg mouse BW. The experimentally confirmed dosing level for the BSA protein in the CDS was 44 mg/kg mouse BW.

These data establish the dose levels and demonstrate the stability of the test and control dosing solutions used in a mouse oral acute toxicity study.

12.0 References

Burzio, L.A. and E. Rice, 2008. Preparation, formulation, and confirmation of dose solutions for an acute oral toxicity study in mice with cold shock protein B. (Study No. CRO-2007-182). Monsanto Technical Report, St. Louis, MSL0021139.

Table 1. Homogeneity of the TDS1 Based on Total Protein Concentration by Amino Acid Analysis[1]

Sample	**Aliquot**	**Observed Concentration** (mg/ml)	**Average Protein Concentration** (mg/ml)	**Overall Mean** (mg/ml)	**SD**	**% CV**
Top	1	0.2838	0.291			
	2	0.2673				
	3	0.3006				
	4	0.3018				
	5	0.3018				
Middle	1	0.2787	0.276	0.286	0.013	4.5
	2	0.2766				
	3	0.2748				
	4	0.2748				
	5	n/d[2]				
Bottom	1	0.2874	0.289			
	2	0.2841				
	3	0.3003				
	4	0.3036				
	5	0.2676				

[1]Homogeneity samples were taken immediately prior to dosing.

[2]n/d = not determined.

Table 2. Homogeneity of the TDS2 Based on Total Protein Concentration by Amino Acid Analysis[1]

Sample	**Aliquot**	**Observed Concentration** (mg/ml)	**Average Protein Concentration** (mg/ml)	**Overall Mean** (mg/ml)	**SD**	**% CV**
Top	1	1.368	1.39	1.44	0.06	3.8
	2	1.328				
	3	1.468				
	4	1.387				
	5	1.414				
Middle	1	1.525	1.49			
	2	1.441				
	3	1.515				
	4	1.481				
	5	n/d[2]				
Bottom	1	1.499	1.46			
	2	1.522				
	3	n/d[2]				
	4	1.412				
	5	1.41				

[1]Homogeneity samples were taken immediately prior to dosing.
[2]n/d = not determined.

Table 3. Observed Total Protein Concentrations of the TDS1 Samples Based on Amino Acid Analysis

Sample	**Aliquot**	**Observed Concentration** (mg/ml)	**Pre- or Post-dose Average Protein Concentration** (mg/ml)	**SD**	**%CV**	**Average** of **Pre- and Post-dose Concentration** (mg/ml)
Pre-dose	1	0.254	0.258	0.0247	9.0	0.276
	2	0.263				
	3	0.266				
	4	0.249				
	5	n/d[1]				
Post-dose	1	0.282	0.294			
	2	0.255				
	3	0.306				
	4	0.316				
	5	0.308				

[1]n/d = not determined.

Table 4. Observed Total Protein Concentrations of the TDS2 Samples Based on Amino Acid Analysis

Sample	**Aliquot**	**Observed Concentration** (mg/ml)	**Pre- or Post-dose Average Protein Concentration** (mg/ml)	**SD**	**%CV**	**Average of Pre- and Post-dose Concentration** (mg/ml)
Pre-dose	1	1.592	1.543	0.086	5.6	1.54
	2	1.624				
	3	1.606				
	4	1.509				
	5	1.384				
Post-dose	1	1.602	1.528			
	2	1.47				
	3	1.646				
	4	1.435				
	5	1.486				

Table 5. Observed Total Protein Concentrations of the CDS Samples Based on Amino Acid Analysis

Sample	**Aliquot**	**Observed Concentration** (mg/ml)	**Pre- or Post-dose Average Protein Concentration** (mg/ml)	**SD**	**%CV**	**Average of Pre- and Post-dose Concentration** (mg/ml)
Pre-dose	1	1.588	1.641	0.035	2.1	1.64
	2	1.694				
	3	1.679				
	4	1.603				
	5	n/d[1]				
Post-dose	1	1.644	1.645			
	2	1.654				
	3	1.621				
	4	1.622				
	5	1.685				

[1]n/d = not determined.

Table 6. Observed Protein Purities of the TDS1, TDS2 and CDS Samples Based on SDS-PAGE and Densitometric Analysis

Dose Identification	**Sample**	**Observed Purity** (%)[1]	**Percent Change**[2]	**Purity** (%) **Used in Dose Calculation**[3]
TDS1	Pre-dose	48.9	1.6	49.3
	Post-dose	49.7		
TDS2	Pre-dose	71.3	4.3	72.9
	Post-dose	74.4		
CDS	Pre-dose	80.6	1.0	80.2
	Post-dose	79.8		

[1]For TDS1, each value represents the mean of purity values estimated from 3 duplicated loadings of 2, 3, and 4 μg of total protein, while for TDS2 and CDS each value represents the mean of purity values estimated from 3 duplicated loadings of 1, 2, and 3 μg of total protein.

[2]Calculated as follows: $\left|\frac{(\text{Pre - dose}) - (\text{Post - dose})}{(\text{Pre - dose})}\right| \times 100\%$

[3]Calculated as the mean of the pre-dose and post-dose protein purity values for each protein.

Table 7. Experimentally Determined Dose Levels[1]

Dose Identification	**Purity Corrected Concentration** (mg/ml)	**Dose Level** (mg/kg BW)
Avg. Dose TDS1	0.14	4.66
Avg. Dose TDS2	1.12	37.3
Avg. Dose CDS	1.32	44.0

[1]TDS1, TDS2 and CDS dose levels were calculated as described in Section 10.6

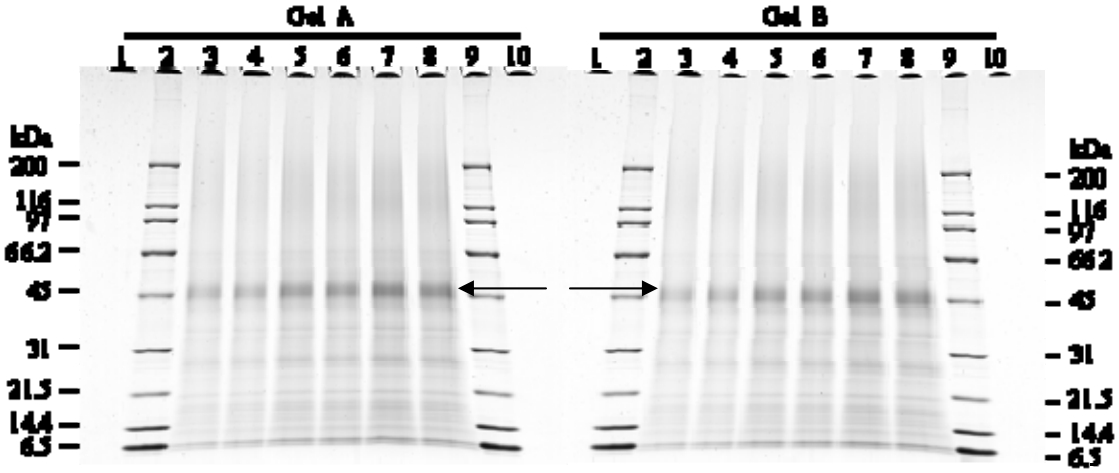

Figure 1. Purity and Molecular Weight Analysis of Pre-Dose and Post-Dose TDS1 Samples

Aliquots of TDS1 samples taken at pre-dose (Gel A) and post-dose (Gel B) were separated on 4-20% polyacrylamide gel and stained with Colloidal Coomassie Blue G. Approximate molecular weights (kDa) are shown on the left and right which correspond to the marker bands in Lanes 1 and 8. Arrows point to the position of the protein of interest.

Lane	Sample	Amount loaded (μg)
1	Empty	
2	BioRad Broad Range Marker	9
3	TDS1 (10001532-D)	2
4	TDS1 (10001532-D)	2
5	TDS1 (10001532-D)	3
6	TDS1 (10001532-D)	3
7	TDS1 (10001532-D)	4
8	TDS1 (10001532-D)	4
9	BioRad Broad Range Marker	9
10	Empty	

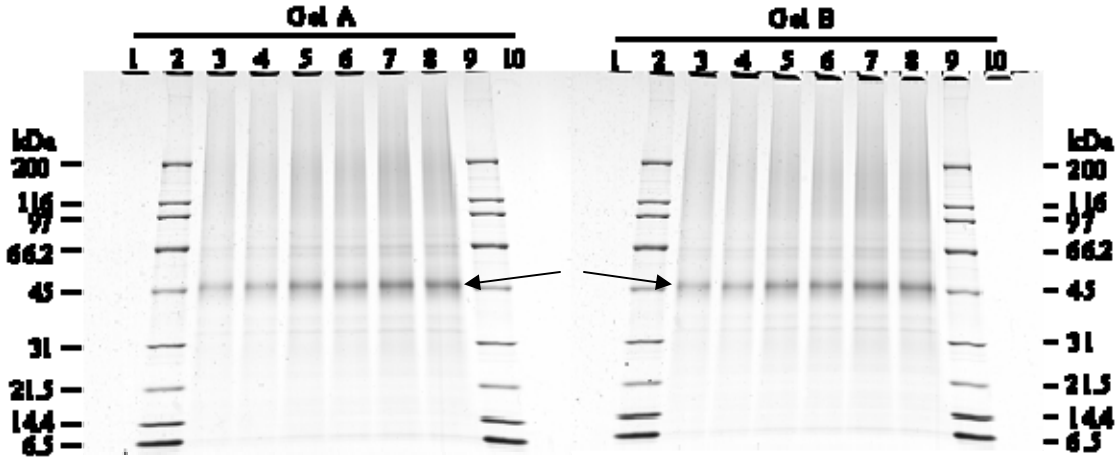

Figure 2. Purity and Molecular Weight Analysis of Pre-Dose and Post-Dose TDS2 Samples

Aliquots of TDS2 samples taken at pre-dose (Gel A) and post-dose (Gel B) were separated on 4-20% polyacrylamide gel and stained with Colloidal Coomassie Blue G. Approximate molecular weights (kDa) are shown on the left and right which correspond to the marker bands in Lanes 1 and 8. Arrows point to the position of the protein of interest.

Lane	Sample	Amount loaded (µg)
1	Empty	
2	BioRad Broad Range Marker	9
3	TDS2 (10001516-D)	1
4	TDS2 (10001516-D)	1
5	TDS2 (10001516-D)	2
6	TDS2 (10001516-D)	2
7	TDS2 (10001516-D)	3
8	TDS2 (10001516-D)	3
9	BioRad Broad Range Marker	9
10	Empty	

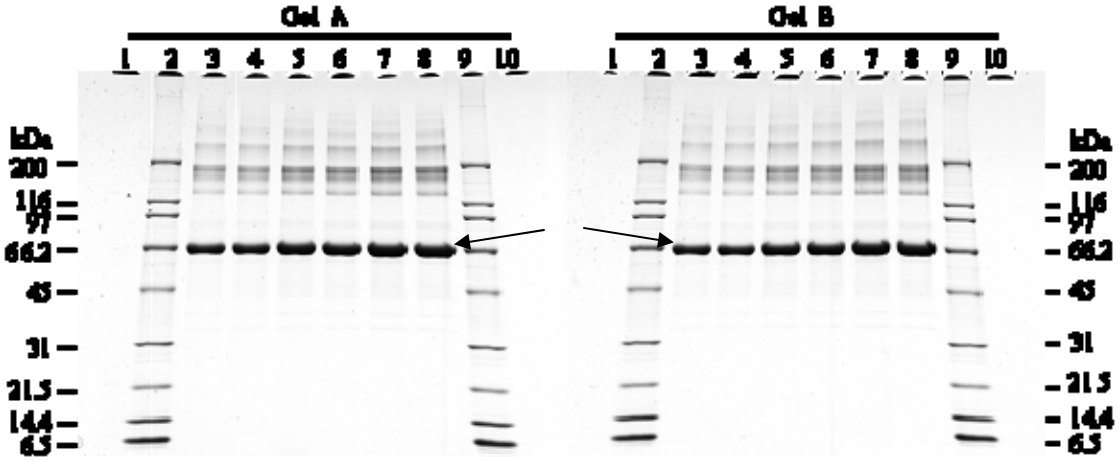

Figure 3. Purity and Molecular Weight Analysis of Pre-Dose and Post-Dose CDS samples

Aliquots of CDS samples taken at the pre-dose (Gel A) and post-dose (Gel B) were separated on 4-20% polyacrylamide gel and stained with Colloidal Coomassie Blue G. Approximate molecular weights (kDa) are shown on the left and right which correspond to the marker bands in Lanes 1 and 8. Arrows point to the position of the protein of interest.

Lane	Sample	Amount loaded (µg)
1	Empty	
2	BioRad Broad Range Marker	9
3	CDS (19305-C)	1
4	CDS (19305-C)	1
5	CDS (19305-C)	2
6	CDS (19305-C)	2
7	CDS (19305-C)	3
8	CDS (19305-C)	3
9	BioRad Broad Range Marker	9
10	Empty	

Appendix 1. List of Applicable SOPs

BR-EQ-0376-02	Hitachi L-8800 Amino Acid Analysis System
BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0525-01	Bio-Rad Protein Assay
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-EQ-0599-02	Bio-Rad GS-710 and GS-800 Densitometers
BR-ME-0956-02	Protein Percent Purity and Apparent Molecular Weight Determination
BR-ME-0973-01	Drying of Polyacrylamide Mini-Gels Using Invitrogen Gel Drying System (Adaptation of Invitrogen Gel Drying Procedure)
BR-ME-0044-03	Diet Incorporation Insect Bioassay for Use in Determining Biological Activity
BR-ME-0990-01	Vapor Phase Acid Hydrolysis Using 6N HCl and Subsequent Amino Acid Analysis

Appendix 3
Detailed Clinical Observation Parameters

Detailed Clinical Observation Parameters

Cage-side Observations	Recorded As
Abnormal movements or behavior	See Categorical
Resistance to removal from cage	Score

Hand-held Observations	Recorded As
Palpebral closure	Score
Lacrimation (non-colored periocular wetness)	Score
Pupil Size	Score
Salivation (non-colored perioral wetness)	Score
Muscle tone	Score
Extensor-thrust response	Score
Reactivity to handling	Score

Open-field observations	Recorded As
Responsiveness to touch	Score
Gait evaluation	Score

Categorical observations (anytime during the DCO)	Recorded As
Abnormal behavior	Description
Abnormalities of the eye	Description
Abnormal urine or feces	Description
Abnormalites of the gastrointestinal (GI) tract	Description
Injury	Description
Missing extremity	Description
Abnormal muscle movements	Description
Palpable mass/swellings	Description
Abnormal posture	Description
Abnormalities of the reproductive system	Description
Abnormal respiration	Description
Abnormal skin or hair-coat/mucous membranes	Description
Excessive soiling	Description
General abnormalities	Description

Explicitly Defined Scales for DCOs

DCO Examination Conduct

The clinical examination is conducted in a careful and systematic format. The examination begins at the head of the animal and gradually works towards the tail as outlined below.

Cage-side observations are made first.

Categorical observations include: Unusual body movements (e.g., tremors, convulsions), abnormal behaviors (e.g., circling, stereotypy) and changes in posture (e.g., arched back, splayed stance).

Resistance to Removal: The degree to which the animal attempts to escape capture is scored. The observer will slowly present a gloved hand into the cage and will grasp the animal over the shoulder area or by the tail.

1 = Decrease – clearly less resistance to capture than typical

2 = Typical – minimally to actively avoids capture and may be mildly aggressive

3 = Increase – clearly more resistance to capture than typical and is very aggressive (attempts to bite).

Eye observations: Eyes are bilaterally examined for these effects; however, if a unilateral observation is made, a concurrent observation is not made for the other eye if it is within normal limits.

Palpebral closure:

1 = Closed (50% to completely closed)

2 = Open

3 = Protruding eyes

Pupil size (aided by penlight): Under typical examination conditions (white light), the typical appearance of the pupils in albino animals is complete constriction. Therefore a decrease in pupil size cannot be observed.

0 = Unable to evaluate

1 = Decrease – clearly decreased pupil size compared to typical

2 = Typical – completely constricted pupils

3 = Increase – clearly increased pupil size compared to typical

Lacrimation (clear wetness): Under typical examination conditions, corneal dryness is not observed in rodents, nor are the eyelids excessively wet.

1 = Decrease – extremely dry appearance of cornea

2 = Typical – glistening cornea (moderate dryness or wetness)

3 = Increase – extensive wetness around the eyes

Degree of salivation: Under typical examination conditions, dryness of the oral cavity is not observed in rodents.

1 = Decrease – oral dryness

2 = Typical – limited to moderate perioral wetness, but lips and chin are dry

3 = Increase – extensive wetness around the mouth and lips

Muscle tone: An assessment of muscle tone at the time of the hand-held observations.

1 = Decrease – clearly less muscle tone than typical

2 = Typical – animal is neither very relaxed nor very tense

3 = Increase – clearly more muscle tone than typical

Extensor-thrust response: Extent of reflex response to brisk pushes (by finger) on the plantar surface of the hindfeet.

1 = Decrease – clearly less response than typical

2 = Typical – clearly detectable extensor-thrust response

3 = Increase – clearly more response than typical

Reactivity to handling: The degree to which an animal struggles to get free from hand-held restraint is ranked.

1 = Decrease – very slight or no struggling

2 = Typical – mild to moderate struggling, animal may vocalize

3 = Increase – aggressive escape behavior, may try to bite observer and usually vocalizes

Observations made in the open-field.

Responsiveness to touch: The ventral aspect of the tail is lightly stroked using a finger. Typically, the animal will lift its tail and wrap it around the finger when lightly touched.

1 = Decrease – does not lift tail, but may briefly hold tail in the air when manually lifted; no response to touch

2 = Typical – lifts tail when touched

3 = Increase – lifts tail and acts startled, may turn towards finger in an attack response

Gait evaluation: Open-field observations are used for gait evaluation. If the animal remains motionless in the open-field, it may be forced to walk on its forelegs while the hindlegs are held off the floor of the observation box ("the wheel-barrow test").

1 = Unable to walk

2 = Clear knuckling, stumbling and poor coordination, may include falling and/or dragging of one or more limbs

3 = Typical – smooth and coordinated gait

Categorical Observations: These observations can be made at anytime during the DCOs. For the categories listed below, the observer directly records the positive observation.

1.	Abnormal behavior	Description
2.	Abnormalities of the eye	Description
3.	Abnormal urine or feces	Description
4.	Abnormalities of the gastrointestinal tract	Description
5.	Injury	Description
6.	Missing extremity	Description
7.	Abnormal muscle movements	Description
8.	Palpable mass/swellings	Description
9.	Abnormal posture	Description
10.	Abnormalities of the reproductive system	Description
11.	Abnormal respiration	Description
12.	Abnormal skin or hair coat/mucous membranes	Description
13.	Excessive soiling	Description
14.	General abnormalities	Description

Appendix 4
Individual Survival and Clinical Observations

(Positive Findings)

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES GROUP 1: 35 MG/KG 22-FEB-08 to 7-MAR-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
8393	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8394	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8395	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8396	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8397	ACTIVITY	0	22-FEB-08	15:19	TREMORS - INTERMITTENT
	BODY	0	22-FEB-08	15:20	URINE STAIN
	BODY	0	22-FEB-08	16:30	URINE STAIN
	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8398	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8399	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8400	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8401	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8402	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES GROUP 2: 1.75 MG/KG 22-FEB-08 to 7-MAR-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
8403	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8404	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8405	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8406	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8407	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8408	BODY	3	25-FEB-08	13:55	UNKEMPT APPEARANCE
	BODY	4	26-FEB-08	15:03	UNKEMPT APPEARANCE
	BODY	9	2-MAR-08	08:39	UNKEMPT APPEARANCE
	BODY	10	3-MAR-08	13:17	UNKEMPT APPEARANCE
	BODY	11	4-MAR-08	13:30	UNKEMPT APPEARANCE
	BODY	12	5-MAR-08	12:44	URINE STAIN
	BODY	12	5-MAR-08	12:44	UNKEMPT APPEARANCE
	BODY	13	6-MAR-08	09:39	UNKEMPT APPEARANCE
	BODY	13	6-MAR-08	09:39	URINE STAIN
	BODY	14	7-MAR-08	08:16	URINE STAIN
	BODY	14	7-MAR-08	08:16	UNKEMPT APPEARANCE
	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8409	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8410	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8411	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES GROUP 2: 1.75 MG/KG 22-FEB-08 to 7-MAR-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
8412	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES GROUP 3: 35 MG/KG 22-FEB-08 to 7-MAR-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
8413	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8414	BODY	10	3-MAR-08	13:22	UNKEMPT APPEARANCE
	BODY	11	4-MAR-08	13:35	UNKEMPT APPEARANCE
	BODY	12	5-MAR-08	12:54	UNKEMPT APPEARANCE
	BODY	13	6-MAR-08	09:46	UNKEMPT APPEARANCE
	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8415	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8416	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8417	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8418	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8419	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8420	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8421	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8422	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES GROUP 1: 35 MG/KG 22-FEB-08 to 7-MAR-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
8423	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8424	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8425	DEAD	14	7-MAR-08	08:55	SCHEDULED EUTHANASIA
8426	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8427	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8428	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8429	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8430	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8431	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8432	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES GROUP 2: 1.75 MG/KG 22-FEB-08 to 7-MAR-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
8433	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8434	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8435	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8436	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8437	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8438	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8439	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8440	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8441	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA
8442	DEAD	14	7-MAR-08	08:56	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES GROUP 3: 35 MG/KG 22-FEB-08 to 7-MAR-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
8443	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8444	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8445	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8446	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8447	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8448	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8449	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8450	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8451	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8452	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA

Appendix 5
Individual Body Weight Data

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES GROUP 1: 35 MG/KG

ANIMAL#	DAY OF STUDY 0 NON-FASTED	0 FASTED	7	14
8393	32.5	29.7	33.4	35.3
8394	28.8	27.9	30.9	31.9
8395	28.7	27.1	32.1	33.1
8396	29.6	27.7	31.1	33.5
8397	31.7	29.8	32.3	33.9
8398	30.8	29.6	32.7	34.7
8399	31.9	29.8	30.8	34.5
8400	32.3	30.3	33.5	34.1
8401	30.6	29.0	30.7	32.8
8402	32.8	30.6	35.7	37.0
MEAN	31.0	29.1	32.3	34.1
S.D.	1.52	1.19	1.59	1.43
N	10	10	10	10

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES GROUP 2: 1.75 MG/KG

ANIMAL#	DAY OF STUDY 0 NON-FASTED	0 FASTED	7	14
8403	31.0	29.2	34.1	34.8
8404	31.1	29.4	29.3	32.4
8405	30.6	28.5	33.4	34.5
8406	29.8	28.3	26.9	31.0
8407	28.5	26.6	27.4	29.9
8408	32.4	30.3	33.1	35.9
8409	28.9	27.4	32.1	34.8
8410	33.8	31.9	34.0	37.6
8411	32.7	31.5	33.2	36.9
8412	31.8	30.6	31.9	34.0
MEAN	31.1	29.4	31.5	34.2
S.D.	1.69	1.73	2.70	2.46
N	10	10	10	10

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES GROUP 3: 35 MG/KG

ANIMAL#	DAY OF STUDY 0 NON-FASTED	0 FASTED	7	14
8413	31.0	29.0	32.1	32.6
8414	33.2	31.2	35.7	38.2
8415	29.1	27.1	30.9	32.3
8416	29.2	27.1	31.6	33.3
8417	32.5	30.5	33.9	36.1
8418	23.3	23.0	27.3	28.8
8419	31.1	29.1	32.1	34.4
8420	30.7	28.8	31.5	33.8
8421	32.4	30.7	34.2	37.0
8422	31.7	29.3	33.7	35.0
MEAN	30.4	28.6	32.3	34.2
S.D.	2.84	2.39	2.31	2.68
N	10	10	10	10

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES GROUP 1: 35 MG/KG

ANIMAL#	DAY OF STUDY 0 NON-FASTED	0 FASTED	7	14
8423	26.9	26.0	25.6	26.5
8424	27.1	26.0	28.4	28.9
8425	28.8	27.2	28.5	28.6
8426	26.5	24.7	26.0	25.9
8427	26.1	25.4	27.0	27.5
8428	25.1	23.7	26.2	26.3
8429	29.4	28.7	28.2	29.5
8430	28.1	26.3	30.2	31.4
8431	26.5	25.8	27.0	30.5
8432	23.4	22.5	26.4	28.0
MEAN	26.8	25.6	27.4	28.3
S.D.	1.75	1.73	1.44	1.83
N	10	10	10	10

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES GROUP 2: 1.75 MG/KG

ANIMAL#	DAY OF STUDY 0 NON-FASTED	0 FASTED	7	14
8433	27.1	24.9	26.2	27.7
8434	25.4	24.2	26.8	28.8
8435	27.5	26.5	27.3	29.6
8436	29.0	27.4	27.1	28.2
8437	25.8	25.0	26.3	28.6
8438	27.8	25.5	29.2	31.1
8439	28.9	27.0	28.1	28.4
8440	26.3	25.0	28.9	30.1
8441	24.4	22.7	25.6	25.4
8442	26.7	25.1	25.9	24.4
MEAN	26.9	25.3	27.1	28.2
S.D.	1.48	1.38	1.24	2.03
N	10	10	10	10

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES GROUP 3: 35 MG/KG

ANIMAL#	DAY OF STUDY 0 NON-FASTED	0 FASTED	7	14
8443	27.1	26.1	27.9	27.8
8444	26.9	26.0	27.8	27.8
8445	25.4	24.2	26.9	27.8
8446	27.1	25.8	27.2	30.2
8447	28.9	28.0	30.2	29.7
8448	25.6	23.7	27.8	30.4
8449	26.5	24.9	26.3	28.7
8450	27.9	26.2	28.9	31.3
8451	29.5	27.5	30.0	32.0
8452	23.1	21.6	26.0	27.5
MEAN	26.8	25.4	27.9	29.3
S.D.	1.83	1.88	1.43	1.63
N	10	10	10	10

Appendix 6
Individual Body Weight Changes

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES GROUP 1: 35 MG/KG

ANIMAL#	DAY OF STUDY 0-7	7-14
8393	3.7	1.9
8394	3.0	1.0
8395	5.0	1.0
8396	3.4	2.4
8397	2.5	1.6
8398	3.1	2.0
8399	1.0	3.7
8400	3.2	0.6
8401	1.7	2.1
8402	5.1	1.3
MEAN	3.2	1.8
S.D.	1.28	0.89
N	10	10

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES GROUP 2: 1.75 MG/KG

ANIMAL#	DAY OF STUDY 0-7	7-14
8403	4.9	0.7
8404	-0.1	3.1
8405	4.9	1.1
8406	-1.4	4.1
8407	0.8	2.5
8408	2.8	2.8
8409	4.7	2.7
8410	2.1	3.6
8411	1.7	3.7
8412	1.3	2.1
MEAN	2.2	2.6
S.D.	2.17	1.10
N	10	10

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES GROUP 3: 35 MG/KG

ANIMAL#	DAY OF STUDY 0-7	7-14
8413	3.1	0.5
8414	4.5	2.5
8415	3.8	1.4
8416	4.5	1.7
8417	3.4	2.2
8418	4.3	1.5
8419	3.0	2.3
8420	2.7	2.3
8421	3.5	2.8
8422	4.4	1.3
MEAN	3.7	1.8
S.D.	0.68	0.69
N	10	10

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES GROUP 1: 35 MG/KG

ANIMAL#	DAY OF STUDY 0-7	7-14
8423	-0.4	0.9
8424	2.4	0.5
8425	1.3	0.1
8426	1.3	-0.1
8427	1.6	0.5
8428	2.5	0.1
8429	-0.5	1.3
8430	3.9	1.2
8431	1.2	3.5
8432	3.9	1.6
MEAN	1.7	1.0
S.D.	1.51	1.06
N	10	10

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES GROUP 2: 1.75 MG/KG

ANIMAL#	DAY OF STUDY 0-7	7-14
8433	1.3	1.5
8434	2.6	2.0
8435	0.8	2.3
8436	-0.3	1.1
8437	1.3	2.3
8438	3.7	1.9
8439	1.1	0.3
8440	3.9	1.2
8441	2.9	-0.2
8442	0.8	-1.5
MEAN	1.8	1.1
S.D.	1.39	1.23
N	10	10

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES GROUP 3: 35 MG/KG

ANIMAL#	DAY OF STUDY 0-7	7-14
8443	1.8	-0.1
8444	1.8	0.0
8445	2.7	0.9
8446	1.4	3.0
8447	2.2	-0.5
8448	4.1	2.6
8449	1.4	2.4
8450	2.7	2.4
8451	2.5	2.0
8452	4.4	1.5
MEAN	2.5	1.4
S.D.	1.04	1.27
N	10	10

Appendix 7
Individual Food Consumption Data

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES GROUP 1: 35 MG/KG

ANIMAL#	DAY 0-7	OF STUDY 7-14
8393	6.1	6.2
8394	6.4	6.7
8395	6.6	7.2
8396	6.5	6.5
8397	6.7	7.3
8398	6.4	5.9
8399	5.6	5.9
8400	7.2	7.0
8401	5.8	6.0
8402	7.0	7.7
MEAN	6.4	6.6
S.D.	0.50	0.65
N	10	10

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES GROUP 2: 1.75 MG/KG

ANIMAL#	DAY OF STUDY 0-7	7-14
8403	8.0	7.9
8404	5.3	6.3
8405	7.1	
8406	4.6	6.3
8407	5.0	5.9
8408	5.9	7.6
8409	6.3	6.6
8410	6.0	6.9
8411	6.5	7.7
8412	6.0	6.5
MEAN	6.1	6.8
S.D.	1.00	0.70
N	10	9

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES GROUP 3: 35 MG/KG

ANIMAL#	DAY 0-7	OF STUDY 7-14
8413	6.4	6.8
8414	5.9	6.9
8415	5.7	6.2
8416	6.1	6.8
8417	6.8	7.2
8418	4.0	5.4
8419	6.4	6.8
8420	6.6	6.8
8421	7.7	7.8
8422	7.3	7.8
MEAN	6.3	6.8
S.D.	1.02	0.71
N	10	10

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES GROUP 1: 35 MG/KG

ANIMAL#	DAY 0-7	OF STUDY 7-14
8423	4.0	5.2
8424	6.3	6.6
8425	5.0	5.1
8426	4.7	5.6
8427	6.2	6.1
8428	5.5	5.7
8429	4.6	6.2
8430	5.9	6.3
8431	3.5	6.9
8432	5.5	5.7
MEAN	5.1	5.9
S.D.	0.93	0.57
N	10	10

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES GROUP 2: 1.75 MG/KG

ANIMAL#	DAY 0-7	OF STUDY 7-14
8433	5.1	5.5
8434	4.3	5.5
8435	6.2	7.5
8436	4.5	6.1
8437	6.4	7.2
8438	6.3	6.6
8439	5.6	6.2
8440	6.0	5.6
8441	4.8	5.8
8442	5.4	6.0
MEAN	5.4	6.2
S.D.	0.76	0.71
N	10	10

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES GROUP 3: 35 MG/KG

ANIMAL#	DAY OF STUDY 0-7	7-14
8443	4.9	5.6
8444	4.9	5.4
8445	6.1	6.2
8446	5.6	6.8
8447	5.1	5.9
8448	6.4	6.9
8449	5.2	5.9
8450	6.2	7.4
8451	6.1	6.5
8452	6.1	5.8
MEAN	5.7	6.2
S.D.	0.59	0.64
N	10	10

Appendix 8
Individual Gross Necropsy Observations

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES GROUP 1: 35 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
8393	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8394	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8395	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8396	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8397	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8398	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8399	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8400	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8401	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8402	7-MAR-08	14	KIDNEY, CORTEX: CYST(S); PRESENT LEFT, ONE, 0.1 CM DIAMETER, CLEAR FLUID FILLED	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES GROUP 2: 1.75 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
8403	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8404	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8405	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8406	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8407	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8408	7-MAR-08	14	HAIRCOAT: WET MATTING; PRESENT UROGENITAL AND ANOGENITAL AREAS, YELLOW	SCHEDULED EUTHANASIA
8409	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8410	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8411	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8412	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES GROUP 3: 35 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
8413	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8414	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8415	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8416	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8417	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8418	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8419	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8420	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8421	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8422	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES GROUP 1: 35 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
8423	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8424	7-MAR-08	14	OVARY: PERIOVARIAN CYST(S); PRESENT LEFT, 0.3 CM DIAMETER, CLEAR FLUID FILLED	SCHEDULED EUTHANASIA
8425	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8426	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8427	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8428	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8429	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8430	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8431	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8432	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES GROUP 2: 1.75 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
8433	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8434	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8435	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8436	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8437	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8438	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8439	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8440	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8441	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8442	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES GROUP 3: 35 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
8443	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8444	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8445	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8446	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8447	7-MAR-08	14	OVARY: PERIOVARIAN CYST(S); PRESENT BILATERAL, ONE ON EACH, EACH APPROXIMATELY 0.5 CM DIAMETER, CLEAR FLUID FILLED	SCHEDULED EUTHANASIA
8448	7-MAR-08	14	CARCASS: BODY FAT DEPLETION; PRESENT	SCHEDULED EUTHANASIA
8449	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8450	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8451	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8452	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA