HAZLETON
LABORATORIES AMERICA, INC
9200 LEESBURG TURNPIKE VIENNA VIRGINIA 22180 USA

PROTOCOL

1. Study	Rat Teratology Study (Segment II)
2. Purpose	To determine the embryo/fetal toxicity and teratogenic potential of a test material administered by gavage to pregnant rats during the period of fetal organogenesis.
3. Test Material	
A. Identification	T-3352
B. Purity	Assume 100%
C. Characteristics	Information on the methods of synthesis and stability, as well as data on composition or other characteristics which define the test material, are on file with the sponsor.
D. Reserve Samples	Reserve samples of the test and control articles will be taken at initiation and retained. These samples, as well as any remaining test material, will be returned to the sponsor upon issuance of the final report.
4. Compliance	This study will be conducted in compliance with all Food and Drug Administration guidelines including the Good Laboratory Practice Regulations, Federal Register, December 22, 1978.
5. Quality Assurance	The protocol, in-life phase, and the final report will be audited by the Office of Quality Assurance in accordance with standard operating procedures at Hazleton Laboratories.

6. Experimental Design

A. Animals

(1) Species	Rat
(2) Strain/Source	Sprague-Dawley CD®/Charles River Breeding Laboratories, Inc.
(3) Age at Receipt	Sexually mature, 8-10 weeks old (females) and 12-14 weeks old (males). Rats having clinical signs and/or body weight extremes will be culled prior to mating.
(4) Number/Sex	100 females/100 males
(5) Identification	Individual eartags
(6) Husbandry	
(a) Housing	Individual housing, except during mating when one female is placed with one male, in hanging wire cages.
(b) Food	Purina Rodent Laboratory Chow® 5001, ad libitum. Sufficient quantities of food will be retained at study initiation to ensure that the same lot number of feed is available for the duration of the study. Feed is analyzed on a prospective basis for concentrations of specified heavy metals, antibiotics, aflatoxin, pesticides, and nitrosamines.
(c) Water	Tap water, ad libitum. The water is routinely analyzed on a retrospective basis for specified microorganisms, pesticides, heavy metals, alkalinity, and halogens.

(d) Contaminants	The study director and/or the sponsor have considered possible interfering substances potentially present in animal feed and water, including the test material itself or possible structurally related materials as well as the items listed in (b) and (c) above. None of these contaminants are reasonably expected to be present in animal feed or water at levels sufficient to interfere with this study.
(e) Environment	Every attempt will be made to maintain temperatures at 72 ± 6°F with a relative humidity of 50 ± 20%. The project coordinator will be notified immediately if the room temperature exceeds a range of 50-90°F. A 12-hour light-dark cycle will be maintained.
(f) Quarantine	Minimum of two weeks.
(7) Randomization Procedures	Upon observation of sperm or vaginal plug, the females will be assigned to experimental groups using a table of random numbers. All animals will be placed on treatment within a two-week period. Prior to terminal sacrifice and cesarean section on day 20 of gestation, all surviving dams will be assigned random numbers so that all personnel performing cesarean sections and external, visceral, or skeletal examination of the fetuses will be unaware of the dose level from which the animals were derived. The numbers will be assigned by random card draw beginning with the first Group 1 animal and continuing numerically

through Group 4. The order of cesarean sections on any given day will be determined by the random number assignment, beginning with the lowest code number and continuing through the highest for that day. This method will result in cesarean sections being performed in a random manner without knowledge of actual animal identity or dose level. Copies of the coded dam numbers will be forwarded to the project coordinator and to the sponsor by the group leader of the study.

Code numbers will be recorded on the prepartum examination records, fetal identification tags, visceral examination records, and skeletal examination records. Upon completion of all data collection and teratologic evaluations, the permanent animal identification and dose level assignments will be transcribed to all data records.

(8) Justification

Rats are sensitive to a number of agents which are known to be embryotoxic and/or teratogenic. Rats historically have been used in safety evaluation studies of this type and are required by the appropriate regulatory agencies. In addition, background teratology control data are available for rats of the proposed strain and source.

B. Group Designation, Dose Levels, and Dosing Schedule

Group No.	Dose Level* mg/kg	Dosing Schedule Day of Gestation	No. of Mated Female Rats
1 (Control)	0	6-15	25
2 (Low)	1	6-15	25
3 (Mid)	10	6-15	25
4 (High)	30	6-15	25

*Based on individual body weights at each weighing interval during the dosing period. Dose levels will be determined upon review of the pilot teratology study data by the sponsor.

C. Dosing Procedures

(1) Method of Administration — The test material will be administered by oral intubation daily on Day 6 through Day 15 of gestation. The dose administered to each female will be based on the most recently recorded individual animal body weight during the dosing period and will be given at approximately the same time each day.

(2) Reason for Dosing Route — Oral intubation is used because of the relative ease of dosing and assurance of the dose amount.

(3) Preparation of Dosing Solutions/ Suspensions — Solutions/suspensions of each concentration will be prepared fresh weekly in Duke's® corn oil. Vehicle and samples of each dosing solution should be shipped within 48 hours of preparation to:

William C. McCormick, III
Toxicologist
Toxicology Services
Medical Dept./3M
220-2E 3M Center
St. Paul, MN 55101

Freezing is not required.

(4) Analysis of Dosing Solutions/Suspensions

(a) Stability — To be conducted by the sponsor.

(b) Homogeneity — To be conducted by the sponsor.

(c) Routine Analyses — To be conducted by the sponsor.

D. Mating Procedures

(1) Breeding — One male and one female will be placed in a breeding cage until a sufficient number of mated females is obtained (maximum breeding period is two weeks).

(2) Vaginal Examinations — During mating, daily vaginal examinations will be made for the presence and viability of sperm or the presence of a copulatory plug. Day of observation of sperm or plug is Day 0 of gestation.

(3) Disposition of Males — The males will be anesthetized, sacrificed, and discarded without necropsy upon completion of the mating.

E. Observation of Animals (Dams)

(1) Observations — All animals will be examined twice daily for mortality and moribundity.

All animals will be observed once daily throughout gestation for clinical signs including time of onset, degree, and duration of effect. Cageside observations will include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory system, circulatory system, autonomic and central nervous system, somatomotor activity, and behavior.

In addition, careful clinical observations will be made at each weighing interval.

Signs will be recorded as they are observed.

(2) Body Weights

Animals will be weighed on Days 0, 6, 8, 12, 16, and 20 of gestation.

(3) Food Consumption

A measured quantity of food will be provided on gestation day 0, and food consumption will be measured on gestation days 6, 8, 12, 16, and 20. Any food added during the gestation intervals will be recorded.

F. Termination

(1) Sick/Moribund/Found Dead Dams

Dams found dead or sacrificed because of sickness or moribundity will be examined for any grossly abnormal thoracic, abdominal, or pelvic viscera. No abnormal viscera will be retained unless requested by the sponsor. Uteri and ovaries will be examined for implantations and corpora lutea, respectively.

Findings will be recorded.

(2) Dams Showing Signs of Abortion or Premature Delivery	Dams exhibiting these signs will be sacrificed by carbon dioxide inhalation. Any grossly abnormal thoracic, abdominal, or pelvic viscera will be noted. No abnormal viscera will be retained unless requested by the sponsor. Uteri and ovaries will be examined for implantations and corpora lutea, respectively. Findings will be recorded.
(3) Terminal Sacrifice	On Day 20 of gestation, all surviving dams will be weighed and sacrificed by carbon dioxide inhalation. The fetuses will be taken immediately by cesarean section, see Section G.(1) below.

G. Postmortem Procedures

(1) Cesarean Section

A cesarean section will be performed on those females sacrificed on Day 20 of gestation. Any grossly abnormal thoracic, abdominal, or pelvic viscera will be noted. No abnormal viscera will be saved unless requested by the sponsor.

The uterus from each female will be excised, weighed, and examined for the number and placement of uterine implantation sites, number of live and dead fetuses, number of early and late resorbing fetuses, and any abnormalities. The uterus will not be reweighed after the contents have been removed. The ovaries will be examined for the number of corpora lutea.

Findings will be recorded.

(2) Fetal Evaluations

Each fetus will be sexed, weighed, examined for external abnormalities, and identified with a tag.

Approximately one-third of all live fetuses from each litter will be selected and processed for visceral examination by the Wilson Technique for assessing soft tissue development. During visceral examination of the fetuses, particular attention will be paid to the examination of the eyes (size, lens, and retina), palate, and brain (third and lateral ventricles). Stainless steel razor blades will be used for sectioning procedures. Abnormalities will be described as anomalies or variants.

The remaining fetuses will be eviscerated and processed for skeletal examination using the Alizarin Red S staining method. This evaluation will include examination of the skull, long bones, vertebral column, rib cage, extremities, and pectoral and pelvic girdles. Bone alignment and degree of ossification will be assessed. Abnormalities will be described as anomalies or variants.

Fetuses will be selected for visceral or skeletal examination in the following manner:

Beginning at the ovarian end of the left uterine horn and continuing past the cervix into the right uterine horn, the first available fetus will be fixed in Bouin's solution for visceral evaluation. The next two fetuses will be processed for skeletal evaluation. This sequence will be repeated until all available fetuses have been selected.

Dead fetuses will be discarded without examination.

(3) Tissue Preservation

The following tissues from each dam will be preserved in 10% neutral buffered formalin:

ovaries uterus

All fetuses will be retained in alcohol, Bouin's fixative, or glycerine.

7. Final Report

At termination of the study, a final report which includes the following information will be prepared and submitted:

A. Experimental Design and Methods

B. Results

(1) Maternal Data - Arranged by test group and supplied in tabular form.

(a) Data for each animal showing:

- identification number
- disposition (pregnancy status and day of cesarean section or sacrifice)
- food consumption at each interval
- body weight at each weighing interval
- daily clinical signs

(b) Data for each dose level showing:

- number of animals placed on study
- number and percent pregnant
- number and percent surviving to cesarean section on gestation day 20
- number and percent that aborted (including premature delivery)
- mean body weight at each weighing interval
- mean body weight change for gestation days 0-6, 6-8, 6-16, 16-20, 0-20, and 6-20
- mean food consumption at each interval
- mean terminal body weight (individual terminal body weights minus gravid uterine weights)
- mean uterine weights
- summary incidence of clinical signs
- summary incidence of gross pathology

(2) Fetal Data - Arranged by test group and supplied in tabular form.

(a) Data for each litter showing:

- identification number
- number and percent of live fetuses
- number of each sex
- number and percent of late resorbing fetuses
- number and percent of total resorbing fetuses

mean live fetal weight by sex
number and percent of dead fetuses
number and percent of early resorbing fetuses
number of corpora lutea
number of implantations
implantation efficiency $\left(\frac{\text{implantations}}{\text{corpora lutea}} \times 100\right)$

(b) Abnormality data for each litter showing:

identification number
number of fetuses examined for external abnormalities;
  number of fetuses within normal limits
  number of fetuses with a particular external variant
  number of fetuses with a particular external anomaly
number of fetuses examined for skeletal abnormalities;
  number of fetuses within normal limits
  number of fetuses with a particular skeletal variant
  number of fetuses with a particular skeletal anomaly
number of fetuses examined for visceral abnormalities;
  number of fetuses within normal limits
  number of fetuses with a particular visceral variant
  number of fetuses with a particular visceral anomaly
incidence and description of each type of abnormality

(c) Cumulative data for each dose level showing:

identification of each group
number of litters examined
mean number and percent of live fetuses
mean weight of live fetuses by sex
mean fetal viability $\left(\frac{\text{live fetuses}}{\text{implantations}} \times 100\right)$
mean number and percent of dead fetuses
mean number and percent of early resorbing fetuses
mean number and percent of late resorbing fetuses
mean number and percent of total resorbing fetuses
mean number of corpora lutea
mean number of implantations
mean implantation efficiency
number and percent of litters with any external variant
number and percent of litters with any external anomaly
number and percent of litters with a particular external variant
number and percent of litters with a particular external anomaly

number and percent of litters with any skeletal variant
number and percent of litters with any skeletal anomaly
number and percent of litters with a particular skeletal variant
number and percent of litters with a particular skeletal anomaly
number and percent of litters with any visceral variant
number and percent of litters with any visceral anomaly
number and percent of litters with a particular visceral variant
number and percent of litters with a particular visceral anomaly
number and percent of litters with any variant
number and percent of litters with any anomaly

C. Statistical Analyses

The significance of suggestive intergroup difference will be tested using appropriate statistical tests.

Body weight changes and total food consumption of pregnant animals during the treatment and post-treatment phases will be analyzed by one-way ANOVA and fetal weights will be analyzed by one-way ANCOVA. If appropriate, Dunnett's t-test will be performed to identify groups with significantly different means from the control. Heterogeneous data, as determined by Levene's test, will undergo a series of transformations to achieve homogeneity of variances prior to performing the above analyses.

Kruskal-Wallis' nonparametric one-way ANOVA or a modified Fisher-Erwin Exact Test, depending on the nature of the data, will be performed on incidence data. In addition, a nonparametric test for trend will be performed on these data. Incidence data to be analyzed include the following:

(1) Percent males/litter

(2) Percent fetal viability

(3) Percent resorptions

(4) Percent of total fetuses examined with a particular external, visceral, or skeletal anomaly or variant

(5) Percent of total litters examined with a particular anomaly or variant (external, visceral, or skeletal)

(6) Percent of total fetuses examined with any anomaly or variant

(7) Percent of total litters examined with any anomaly or variant

All tests for significance are made at the 5.0% probability level, two-tailed for the group comparisons and one-tailed for Levene's F-tests and trend analysis.

The final report, or a draft copy of such, will be reviewed by the sponsor within three months of receipt. Any revisions or comments will be submitted to Hazleton within this period.

5089B
6/2/83