FINAL REPORT

Study Title

Interlaboratory Validation of the Pubertal Female Assay

Author

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(Study Director)

Study Completed On

3 January 2006
(Final Report)

Performing Laboratory

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Laboratory Project ID

CR-DDS Argus Division Protocol Number: RTP00001

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentially is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10(d) (1)(A), (B), or (C).

This statement supersedes any other claims of confidentiality found in this report.

Company: Battelle

Company Agent: David P. Houchens, Ph.D.

Title: Program Manager

Date: _____

Signature: _____

GOOD LABORATORY PRACTICE STATEMENT

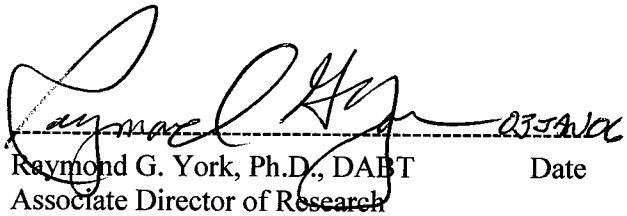
This study was conducted according to U.S. Environmental Protection Agency. Federal Insecticide, Fungicide and Rodenticide Act/Toxic Substances Control Act (FIFRA/TSCA); Good Laboratory Practice Standards; Final Rule. 40 CFR Part 160/Part 792 with the exception that the statistical analysis conducted by BioSTAT Consultants, Inc., was only conducted with quality control procedures and was not subjected to quality assurance audit. Any areas of noncompliance are documented in the study record. No deviations existed that affected the validity of the study.

Submitter:

Sponsor's Representative:

David P. Houchens, Ph.D. Date
Battelle

Study Director:



Raymond G. York, Ph.D., DABT Date
Associate Director of Research
and Study Director

FLAGGING STATEMENT

I have applied the criteria of 40 CFR 158.34 for flagging studies for potential adverse effects to the results of the attached study. This study neither meets nor exceeds any of the applicable criteria.

Company: Battelle

Company Agent: David P. Houchens, Ph.D.

Title: EDSP Program Manager

Date: _____

Signature: _____

TITLE: INTERLABORATORY VALIDATION OF THE PUBERTAL FEMALE ASSAY

CR-DDS ARGUS DIVISION PROTOCOL NUMBER: RTP00001

SPONSOR'S WORK ASSIGNMENT AND TASK NUMBER:
WA 4-14, Task 4

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TITLE: INTERLABORATORY VALIDATION OF THE PUBERTAL FEMALE ASSAY

CR-DDS ARGUS DIVISION PROTOCOL NUMBER: RTP00001

SPONSOR'S WORK ASSIGNMENT AND TASK NUMBER: WA 4-14, TASK 4

ABSTRACT

This study was done as a step toward validating the Female Pubertal Assay (EDSTAC, 1998). The transferability of the protocol was evaluated using methoxychlor, DE-71 and 2-chloronitrobenzene, the first two chemical compounds are known to affect the endocrine system through different pathways and/or mechanisms. This study is expected to detect estrogenic-, androgenic-, and thyrotropic-like activity based on compound-related changes in target organ weight, sexual maturation and systemic hormones.

Juvenile female Sprague-Dawley Crl:CD®(SD) rats, 15/group, were dosed between 0700 and 0900 hours each day for three weeks via oral gavage with methoxychlor at 12.5 and 50 mg/kg/day, DE-71 at 30 and 60 mg/kg/day, or 2-chloronitrobenzene at 25 and 100 mg/kg/day. Dosages were formulated in corn oil as the vehicle and administered at 2.5 mL/kg.

Body weights were recorded before dosing on preweaning days 0, 4, 7, 14 and 21 and daily postweaning (PNDs 21 through 42) and clinical observations were recorded daily within one hour after dosing in the morning and again in the afternoon.

Female rats were evaluated for age and body weight at vaginal patency and estrous cycling was evaluated by vaginal cytology beginning at sexual maturation until sacrifice. Necropsies were performed on PND 42, two hours after the last dose administration and prior to 1300 hours. Trunk blood was collected from anesthetized animals following decapitation for thyroxine (T4) and thyroid stimulating hormone (TSH) analyses. Ovaries, uteri, livers, kidneys, pituitaries and adrenal glands were collected and weighed and histopathology was conducted on the thyroids, ovaries and uteri of all F1 generation female rats.

- **Methoxychlo**r

There were no deaths on the study. There were no significant differences in the mean body weights across the control and two treatment groups on PNDs 21 through 42. There were no clinical or necropsy observations that were treatment-related. Body and organ weights were unaffected by treatment of the test substance. Vaginal opening was significantly accelerated and the average body weight at time of vaginal opening was decreased in the 50 mg/kg/day dosage group. Increased follicular epithelial cell height and colloid depletion was evident in the thyroids from the 50 mg/kg/day dosage group,

indicative of a hypothyroid state. Ovarian and uterine histopathology was normal. There was no change in T_4 or TSH levels.

- **DE-71**

There were no deaths on the study. There were no significant differences in the mean body weights across the control and two treatment groups on PNDs 21 through 42. There were no clinical or necropsy observations that were treatment-related. Liver weights were significantly increased in the 30 and 60 mg/kg/day dosage groups of the test substance. The average age of vaginal opening and average body weight at time of vaginal opening were unaffected by dosages of 30 or 60 mg/kg/day DE-71. A dosage-dependent increase in follicular epithelial cell height and marginal colloid depletion was evident in the thyroids from the 30 and 60 mg/kg/day dosage levels, indicative of a hypothyroid state. Ovarian and uterine histopathology was normal. Serum T4 levels were significantly depressed in the 30 and 60 mg/kg/day dosage groups.

- **2-Chloronitrobenzene**

There were no deaths on the study. There were no significant differences in the mean body weights across the control and two treatment groups on PNDs 21 through 42. A dose dependent increase in excess salivation (slight to extreme) was observed in the 25 and 100 mg/kg/day dosage groups and a black spleen was observed at necropsy in 86.7% of the females in the 100 mg/kg/day dosage group. Liver weights were significantly increased in the 25 and 100 mg/kg/day dosage groups and the absolute mean weight of the uterus (without fluid) was significantly decreased in the 100 mg/kg/day dosage group. The average age of vaginal opening and average body weight at time of vaginal opening were significantly increased in the 100 mg/kg/day 2-chloronitrobenzene dosage group. Increased follicular epithelial cell height and colloid depletion was evident in the thyroids from the 25 and 100 mg/kg/day dosage groups, indicative of a hypothyroid state. Ovarian and uterine histopathology was normal. There was no change in T_4 or TSH levels.

1. OBJECTIVES

The purpose of this project is to quantify the effects of chemicals on pubertal development and thyroid function in the intact juvenile/peripubertal female rat. This assay detects chemicals that display anti-thyroid, estrogenic/antiestrogenic, or alter hypothalamic function or luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin, or growth hormone (GH) secretion.

The EPA has selected three test chemicals for evaluation in the female pubertal assay validation, and has selected the low and high target doses (in mg/kg/day) for each of them. The three test chemicals and their target/mechanism of action are as follows: (1) methoxychlor - a xeno-estrogen through the α-estrogen receptor, an anti-estrogen through the β-estrogen receptor, and an anti-androgen through an androgen receptor-mediated mechanism; (2) DE-71, a commercial mixture of polybrominated diphenyl

ethers – a thyroid active chemical that increases clearance of thyroxine (T4) through induction of hepatic microsomal phase II enzyme uridine diphospho-glucuronosyl transferase (UDPGT) activity; and (3) 2-chloronitrobenzene – which was presumed to have no endocrine effects.

2. DESCRIPTION OF TEST PROCEDURES

2.1. Conduct of Study

2.1.1. Sponsor

Battelle, 505 King Avenue, Columbus, Ohio 43201-2693, USA

2.1.2. Testing Facility

Charles River Laboratories Discovery and Development Services (CR-DDS), Argus Division, 905 Sheehy Drive, Building A, Horsham, PA 19044-1241, USA

2.1.3. Study Number

RTP00001

2.1.4. Sponsor's Work Assignment and Task Number

WA 4-14, Task 4

2.1.5. Purpose of the Study

The purpose of this study was to investigate the transferability of a protocol designed to quantify effects of chemicals on pubertal development and thyroid function in the juvenile/peripubertal female rat. This assay detected chemicals that display anti-thyroid or estrogenic/anti-estrogenic activity [estrogen receptor (ER) or steroid enzyme mediated], or alter hypothalamic function or luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin, or growth hormone (GH) secretion.

2.1.6. Regulatory Compliance

This study was conducted in compliance with Good Laboratory Practice (GLP) regulations of the Environmental Protection Agency [(1)]. Quality Assurance Unit findings derived from the inspections during the conduct of this study are documented and have been provided to the Study Director and the Testing Facility Management.

2.1.7. Ownership of the Study

The U.S. Environmental Protection Agency owns the study. All raw data, analyses, reports and preserved tissues are the property of the U.S. Environmental Protection Agency.

2.1.8. Work Assignment Manager

James P. Kariya, M.S. (U.S. Environmental Protection Agency, Endocrine Disruptor Screening Program)

2.1.9. Work Assignment Leader

Jerry D. Johnson, Ph.D., DABT
Address as cited previously for Sponsor

2.1.10. Program Manager

David P. Houchens, Ph.D. (Program Manager)
Address as cited previously for Sponsor

2.1.11. Study Director

Raymond G. York, Ph.D., DABT (Associate Director of Research)
Address as cited previously for Testing Facility.

2.1.12. Technical Performance - CR-DDS

John F. Barnett, B.S. (Director of Operations, Argus Division)
Gerard M. Zimmerman, ALAT (Study Supervisor, Argus Division)
Daniel E. Fisher, B.S. (Laboratory Technician, Argus Division)
Giovanni D. Brooks, B.S. (Necropsy Laboratory Technician, Argus Division
Kevin E. Cegielski (Formulation Laboratory Technician, Argus Division)
Julian Gulbinski III, B.S., M.B.A. (Scientist, Argus Division) - Thyroxine analyses
Melissa A. Snyder, B.S. (Formulation Laboratory Technician, Argus Division) - Thyroxine analyses
Richard Norlin, M.S. (Principal Investigator, Worcester Division) - Formulation Analyses
John M. Pletcher, DVM, MPH, DACVP (Principal Investigator, Pathology Associates Division) - Histopathology Analyses

2.1.13. Battelle

Michael E. Cobb (Batelle Marine Sciences Laboratory, Sequim, Washington, USA) - Bulk Test Substance Analyses

2.1.14. BioSTAT Consultants, Inc.

BioSTAT Consultants, Inc., Portage, Michigan, USA - Statistical Analyses

2.1.15. CTBR Bio-Research Inc.

Khaldoon Abuarjah, BSc, MT(ASCP) (Principal Investigator, CTBR Bio-Research Inc., Senneville, Quebec, Canada) - Thyroid Stimulating Hormone (TSH) Analyses

2.1.16. Report Preparation

Raymond G. York, Ph.D., DABT
Joseph W. Lech, B.S., LAT (Scientist)
Scott A. Johnson, B.S. (Data Management Specialist)
Tsai-Liang Chiang, B.S. (Senior Report Administrator)

2.1.17. Report Review

Alan M. Hoberman, Ph.D., DABT (Director of Research)
Valerie A. Sharper, M.S. (Principal Research Scientist)

2.1.18. Date Protocol Signed

5 March 2005

2.1.19. Dates of Technical Performance

P Generation Rat Arrival	22 FEB 05
Delivery Period (PND[a] 0)	08 MAR 05 - 09 MAR 05
PND 4 Sacrifice	12 MAR 05 - 13 MAR 05
PND 21 Sacrifice (Dams and F1 generation pups not selected for continued observation)	29 MAR 05 - 30 MAR 05
Dosage Period (PND 22 through PND 42)	30 MAR 05 - 20 APR 05
Sexual Maturation (VO[b]) Evaluation	30 MAR 05 - 17 APR 05
PND 42 Sacrifice (Scheduled sacrifice F1 generation rats)	19 APR 05 - 20 APR 05

2.1.20. Records Maintained

The original report, raw data and reserve samples of the bulk test substance and bulk vehicle are retained in the archives of the Testing Facility. Any preserved tissues are retained in the archives of the Testing Facility for one year after the mailing of the draft final report, after which time the Sponsor will decide their final disposition. All residual formulations were discarded at the Testing Facility. Backup samples will be discarded at

a. PND is used as an abbreviation for postnatal day, day postpartum or day of lactation. PND 0 is defined as the day of birth.
b. VO is used as an abbreviation for vaginal opening.

the Testing Facility following issue of the final report. Disposition of the remaining bulk test substances will be documented in the raw data.

2.2. Test Substance Information

Note: The Sponsor provided the test substances. Except for chemistry formulation and analyses, all tests, analyses and measurements were conducted by individuals without knowledge of the identity of the test substances. A key code for the dosage levels and concentrations were provided to the formulation and Quality Assurance personnel for the purpose of formulation preparation and auditing of critical phases, respectively. The identities of the test substances dosage levels and concentrations were added to the protocol by amendment following the completion of the in-life phases of the study

2.2.1. Descriptions, Dates Received, Storage Conditions, Lot Numbers and Expiration Dates

Test Substance (CAS No.)	Description	Date Received	Storage Conditions	Lot Number	Expiration Date
Methoxychlor (72-43-5)	Faint orange powder	16 DEC 04	Room temperature	102K1373	13 OCT 09
DE-71 (32534-81-9)	Viscous amber liquid	15 DEC 04	Room temperature	4550OD23D	03 NOV 10
2-Chloronitrobenzene (88-73-3)	Yellow crystalline solid	21 DEC 04	Room temperature in a tightly closed container	09019MC	01 NOV 10

2.2.2. Special Handling Instructions

Standard safety precautions (use of protective clothing, gloves, tyvek® sleeves or tyvek® suit, dust-mist/HEPA-filtered mask, safety goggles or safety glasses with side shields) were taken during formulation preparation and dosage. The bulk test substances were handled in a chemical fume hood.

2.2.3. Analysis of Activity

A Certificate of Analysis for each test substance is available in APPENDIX 8. The Sponsor's signature and approval of the protocol indicates that appropriate documentation of the method of synthesis, fabrication or derivation of the test substances is on file and that it is available to the appropriate regulatory agencies should it be requested. Information to document or certify the identity, composition, strength and activity of each test substance was generated on study EDSP. 414-01 and provided by the Sponsor to the Testing Facility. The results from these analyses are available in APPENDIX 9.

2.3. Vehicle Information

2.3.1. Description

Corn Oil - a clear, yellow liquid

2.3.2. Lot Numbers

AO-001
AO-002
AO-003[a]

2.3.3. Dates Received and Storage Conditions[b]

The vehicle was received from the Sponsor on 17 December 2004 (AO-001) and 26 January 2005 (AO-002). Lot AO-001 was stored at room temperature from 17 December 2004 through 17 January 2005; it was stored refrigerated beginning on 17 January 2005 at the Sponsor's request. Lot AO-002 was stored refrigerated.

2.3.4. Special Handling Instructions

Standard safety precautions (use of protective clothing, gloves, dust-mist/HEPA-filtered mask, safety goggles or safety glasses with side shields) were taken when handling the vehicle.

2.3.5. Analysis of Activity/Purity

Neither the Sponsor nor the Study Director was aware of any potential contaminants likely to have been present in the vehicle that would have interfered with the results of this study. The peroxide content of the corn oil used for preparing the formulation was analyzed to make certain that the peroxide content is <3 mEq/mL[c].

2.4. Test Substance Preparation and Storage Conditions

Solutions for administration were prepared once at the Testing Facility. Prepared solutions were stored refrigerated.

Prior to study start, the Testing Facility performed a pre-study preparation and analysis of the test substance formulations in order to validate the transfer of information provided by the Sponsor regarding preparation and analysis of the test substance formulations.

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a. See PROTOCOL DEVIATIONS, item 1.
b. At the Sponsors request the corn oil from lots AO-001 and AO-002 were combined in a 50L carboy and thoroughly mixed. As a result, the combined lot of corn oil was assigned lot number of AO-003 by the Testing Facility.
c. See PROTOCOL DEVIATIONS, items 2 and 3.

2.4.1. Sample Information

Sample Type	Size	Date Retained	Storage Conditions	Shipped To/Shipping Conditions	Date Shipped
Methoxychlor					
Bulk Test Substance[a]	1 g	20 APR 05	Room temperature	Sponsor[b]/Room temperature	20 APR 05
Concentration[c] (all levels)	1 mL	11 MAR 05 21 MAR 05	Refrigerated	CRL-DDS Worcester Division[d]/Refrigerated	11 MAR 05 22 MAR 05
Bulk Test Substance Reserve	1 g	11 MAY 05	Room temperature	Testing Facility Archives	17 MAY 05
DE-71					
Bulk Test Article/Substance[a]	1g	20 APR 05	Room temperature	Sponsor[b]/Room temperature	20 APR 05
Concentration[e] (all levels)	1 mL	18 MAR 05 21 MAR 05	Refrigerated	CRL-DDS Worcester Division[d]/Refrigerated	18 MAR 05 22 MAR 05
Bulk Test Substance Reserve	1 g	11 MAY 05	Room temperature	Testing Facility Archives	17 MAY 05
2-Chloronitrobenzene					
Bulk Test Substance[a]	1 g	20 APR 05	Room temperature	Sponsor[b]/Room temperature	20 APR 05
Concentration[f] (all levels)	1 mL	10 MAR 05 21 MAR 05	Refrigerated	CRL-DDS Worcester Division[d]/Refrigerated	11 MAR 05 22 MAR 05
Bulk Test Substance Reserve	1 g	11 MAY 05	Room temperature	Testing Facility Archives	17 MAY 05
Vehicle Reserve[g]	1g	11 MAY 05	Refrigerated	Testing Facility Archives	17 MAY 05

a. A sample of the bulk test substance was taken on the last day of treatment and shipped for analysis.
b. Battelle Marine Sciences Laboratory, Sequim, Washington, USA.
c. Six samples were taken from each preparation on the day prepared from the methoxychlor formulations in order to: 1) validate the transfer of information provided by the Sponsor from the pre-study preparation and 2) verify the concentration of the test substance in the vehicle from the dosing formulations. Three samples from each set were shipped for analysis; the remaining samples were retained at the Testing Facility as backup samples.
d. CR-DDS Worcester Division, Worcester, Massachusetts, USA.
e. Quadruplicate samples were taken from each preparation on the day prepared from the DE-71 formulations for both RTP00001 and RTP00002 in order to: 1) validate the transfer of information provided by the Sponsor from the pre-study preparation and 2) verify the concentration of the test substance in the vehicle from the dosing formulations. Two samples from each quadruplicate set were shipped for analysis; the remaining samples were retained at the Testing Facility as backup samples.
f. Six samples were taken from each preparation on the day prepared from the 2-chloronitrobenzene formulations for both RTP00001 and RTP00002 in order to: 1) validate the transfer of information provided by the Sponsor from the pre-study preparation and 2) verify the concentration of the test substance in the vehicle from the dosing formulations. Three samples from each set were shipped for analysis; the remaining samples were retained at the Testing Facility as backup samples.
g. See PROTOCOL DEVIATIONS, item 4.

2.4.2. Formulation Analyses

Information to document the stability of the prepared formulations bracketing the range of concentrations used in this study was provided by the Sponsor and is available in APPENDIX 9. Information to document the solubility of methoxychlor, DE-71 and 2-chloronitrobenzene in the vehicle over the range of concentrations used in this study was provided by the Sponsor and is available in APPENDIX 9. The results of the concentration results for all test substances used on study are available in APPENDIX 9. The concentration results from the start of study concentration results for the DE-71 are located in the Method Validation and Formulation Sample Analysis report for study RTP00002 located in APPENDIX 9.

2.5. Test System

2.5.1. Species

Rat

2.5.2. Strain

Crl:CD(SD)

2.5.3. Supplier (Source)

Charles River Laboratories, Inc., Portage, Michigan, USA (P generation). The F1 generation female rats assigned to study were delivered at the Testing Facility.

2.5.4. Sex

2.5.4.1. P Generation

Female (NOTE: P generation dams were provided by the Supplier to maintain the F1 generation pups and were not considered part of the Test System.) Body weights ranged from 229g - 280g on the day following arrival.

2.5.4.2. F1 Generation

Female

2.5.5. Rationale for Test System

The Crl:CD(SD) rat was selected as the Test System because of known response to toxic effects on reproductive capacity and history of use as a rodent species in these evaluations[2-4].

2.5.6. Test System Data

Number of F1 generation Rats	105
Dates of Birth	08 MAR 05 - 09 MAR 05
Weight (g) at Study Assignment	44.6 - 57.3

2.5.7. Method of Randomization

2.5.7.1. P Generation Rats

Upon arrival, P generation rats were assigned to individual housing on the basis of computer-generated random units.

2.5.7.2. F1 Generation Pups/Rats

On PND 4, litters were standardized, to consist of at least eight and a maximum ten pups per litter, maximizing the number of females left in the litter and retaining males as necessary to reach the eight to ten pups per litter to be continued on study. Litters with fewer than eight pups were not retained. Unthrifty or runted pups were excluded from the study.

On PND 21 before the first administration, 105 F1 generation female rats were selected for study. Rats were marked by litter and individually weighed to the nearest 0.1 g and ranked by body weight. A population of rats that was as homogeneous as possible were selected for study by eliminating an equal number of pups from the heavy end and the light end of distribution, leaving the number of rats needed for study in the middle. Female rats were assigned to treatment groups such that the mean body weights and variances for all groups are similar. Littermates were not assigned to the same dosage group.

The pups assigned to study derived from litters delivered over two consecutive days. Assignment to study on PND 21 was therefore conducted over two consecutive days.

On PND 21 female pups were temporarily tail-marked with a Sharpie® marker with the numbers 01, 02, etc., through the nth female in each litter. The pup's weights were then recorded into a computer file that identifies each female pup by a unique temporary number that combines the last 2 digits of the dam's number with the pup's number. For instance the first 3 female pups to be weighed from litter 5901 were labeled 101, 102, and 103, and the first 3 pups from litter 5919 were labeled 1901, 1902, and 1903.

Following collection of the PND 21 pup weights, a body weight-ordered listing of the pups was generated. No single dosage group was to contain siblings, thereby potentially compromising capability to exclude body weight outliers from study assignment. Since no more than seven pups were allowed to be assigned to study from each litter, the lightest and/or heaviest of the 8th through nth female pups from those litters containing more than seven females were considered ineligible for study assignment; those pups

were crossed off of the body weight-ordered list. The day's PND 21 body weight rankings was then examined by the Study Director, who approved any further exclusions for body weight considerations.

A table of random units was then used to assign all eligible pups to dosage groups I through VII; the 7 lightest-weight pups were each be randomly assigned to one of the 7 dosage groups, then the 7 next lightest-weight pups were assigned, and so on until all eligible pups had been assigned.

The dosage group assignment lists were then reviewed to ensure that no dosage group contained siblings. Reassignment of dosage groups was made with consideration given to body weight similarity, in the event of inter-group siblings.

Following any necessary dosage group reassignments, the pups assigned to study were assigned unique permanent numbers and were tail-tattooed prior to weaning them into group housing in nest boxes.

Pups were placed onto study on the basis of clinical observations and body weights recorded on PND 21; supervisory physical examinations of the pups were not conducted. After assignment to treatment groups, rats were housed in groups of three rats per cage, such that each cage has the same number of rats.

This method of selection and assignment to treatment groups is also documented in the raw data.

2.5.8. System of Identification

2.5.8.1. P Generation Rats

Female rats were assigned temporary animal numbers at receipt. The rats were permanently identified using Monel® self-piercing ear tags (Gey Band and Tag Co., Inc., No. MSPT 20101) following natural delivery of the pups. Cage tags were marked with the study number, permanent rat number, sex and generation.

2.5.8.2. F1 Generation Pups/Rats

Pups were not individually identified during the postpartum period; all parameters were evaluated in terms of the litter. On PND 21, pups were marked by litter for randomization to study groups. Each F1 generation rat selected for continued observation was identified by tail tattoo according to the Standard Operating Procedures of the Testing Facility. Cage tags were marked with the study number, permanent rat number, sex, generation and group number.

2.6. Husbandry

2.6.1. Research Facility Registration

USDA Registration No. 14-R-0144 under the Animal Welfare Act, 7 U.S.C. 2131 *et seq*.

2.6.2. Study Room

The study room was maintained under conditions of positive airflow relative to a hallway and independently supplied with a minimum of ten changes per hour of 100% fresh air that had been passed through 99.97% HEPA filters. Room temperature and humidity were monitored constantly throughout the study. Room temperature was targeted at 68ºF to 75ºF (20ºC to 24ºC); relative humidity was targeted at 40% to 50%[a].

2.6.3. Housing

All cage sizes and housing conditions were in compliance with the *Guide for the Care and Use of Laboratory Animals*[5].

2.6.3.1. P Generation Rats/F1 Generation Litters

P generation rats were individually housed in clear plastic nesting boxes (45.7 cm x 25.4 cm x 20.3 cm). Each dam and delivered litter was housed in a common nesting box during the postpartum period.

2.6.3.2. F1 Generation Rats

After weaning, the F1 generation female rats were group housed in nesting boxes (three pups per nesting box) by dosage group.

2.6.4. Light

An automatically controlled 14-hours light:10-hours dark fluorescent light cycle was maintained. Each dark period began at 1900 hours.

2.6.5. Sanitization

Nesting boxes were changed approximately every other week. Bedding was changed as often as necessary to keep the rats dry and clean.

2.6.6. Diet

Rats were given Harlan's Teklad 2018c meal feed, available *ad libitum* from individual feeders.

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a. See APPENDIX 10 (ENVIRONMENTAL AND HUSBANDRY REPORTS).

2.6.7. Diet Analysis

Analyses were performed by the feed supplier. No contaminants at levels exceeding the maximum concentration limits for certified feed or deviations from expected nutritional requirements were detected by these analyses.

The concentrations of genistein equivalents (genistein plus 0.8 x daidzein) were [124.5 + (0.8 x 109.5) = 212.1 ppm which is ≤ 300 ppm per lot. The diet was analyzed by separating the conjugated and unconjugated (aglycone forms) of genistein, daidzein, and glycitein in the diet using high-pressure liquid chromatography (HPLC). Each of those forms was then converted into aglycone equivalents[6]. Copies of the results of the feed analyses are available in the raw data and in APPENDIX 10.

Neither the Sponsor nor the Study Director was aware of any potential contaminants likely to have been present in the feed that would have interfered with the results of this study.

2.6.8. Water

Local water that had been processed by passage through a reverse osmosis membrane (R.O. water) was available to the rats *ad libitum* from individual water bottles attached to the cages. Chlorine was added to the processed water as a bacteriostat.

2.6.9. Water Analysis

The processed water is analyzed twice annually for possible chemical contamination (Lancaster Laboratories, Lancaster, Pennsylvania, USA) and monthly for possible bacterial contamination (QC Laboratories, Southampton, Pennsylvania, USA). Copies of the results of the water analyses are available in the raw data and in APPENDIX 10.

Neither the Sponsor nor the Study Director was aware of any potential contaminants likely to have been present in the water that would have interfered with the results of this study.

2.6.10. Bedding Material

Nesting material (heat-treated laboratory-grade pine shavings) was provided.

Bedding was changed at least once a week or as often as necessary to keep the rats dry and clean. Bedding changes were documented in the raw data.

Corn cob bedding was not used.

2.6.11. Bedding Analysis

Each lot of bedding was analyzed (Lancaster Laboratories, Lancaster, Pennsylvania, USA) for possible contamination. Copies of the results of the bedding analyses are available in the raw data and in APPENDIX 10.

Neither the Sponsor nor the Study Director was aware of any potential contaminants likely to have been present in the bedding that would have interfered with the results of this study.

2.7. Methods

2.7.1. Dosage Administration

Dosage Group	Test Substance/()[a]	Dosage[b] (mg/kg/day)	Concentration (mg/mL)	Dosage Volume (mL/kg)	Number of Rats	Assigned F1 Generation Rat Numbers
I	Corn oil (G)	0	0	2.5	15	101 - 115
II	Methoxychlor (A)	12.5	5	2.5	15	116 - 130
III	Methoxychlor (B)	50	20	2.5	15	131 - 145
IV	DE-71 (C)	30	12	2.5	15	146 - 160
V	DE-71 (D)	60	24	2.5	15	161 - 175
VI	2-Chloronitrobenzene (E)	25	10	2.5	15	176 - 190
VII	2-Chloronitrobenzene (F)	100	40	2.5	15	191 - 205

a. Assigned Group Letter
b. The test substance was considered 100% active except methoxychlor which was adjusted for approximately 95% purity for the purpose of dosage calculations.

2.7.2. Rationale for Dosage Selection

Methoxychlor and DE-71 dosages were selected by the Sponsor on the basis of previous study results with the test substances and the intent to replicate these results but not on the Maximum Tolerated Dose (MTD) level. However, the MTD and the 1/4 MTD were used for 2-chloronitrobenzene.

2.7.3. Route and Rationale for Route of Administration

The oral (gavage) route was selected for use because: 1) in comparison with the dietary route**,** the exact dosage can be accurately administered; and 2) it is one possible route of human exposure.

Appropriate needle sizes were used. The needle size used for F1 female rats $< 50 \pm 5$ grams was a 22 gauge stainless steel, 1 or 1.5 inch with a 1.25 mm ball tip needle. The needle size used for F1 female rats $> 50 \pm 5$ grams was a 20 gauge stainless steel, 1.5 inch with a silicone tip, 2 mm ball tip feeding needle.

2.7.4. Frequency of Administration

2.7.4.1. P Generation Rats

P generation rats were not given the test substances and/or vehicle.

2.7.4.2. F1 Generation Rats

F1 generation rats were given the test substances and/or vehicle on PNDs 22 through 42. Dosages were given once daily, between 0700 and 0900 each day[a]. Dosages were adjusted daily for body weight changes and given at approximately the same time each day. Daily dosage volumes were documented.

2.7.5. Method of Study Performance

2.7.5.1. P Generation Rats

Twenty-five timed-mated rats were received at the Testing Facility. Rats were observed for viability at least twice daily. Body weights were recorded weekly during the acclimation period and at sacrifice. Feed was monitored and replenished on an as-needed basis.

2.7.5.2. F1 Generation Pups

PND 0 of was defined as the day of birth and was also the first day on which all pups in a litter are individually weighed (pup body weights will be recorded after all pups in a litter are delivered and groomed by the dam).

Each litter was evaluated for viability at least twice daily. The pups in each litter were counted once daily. Clinical observations were recorded once daily during the preweaning period. Pup body weights were recorded on PNDs 0, 4, 7, 14 and 21.

2.7.5.3. F1 Generation Rats

NOTE: Test substances provided by the Sponsor were identified by code. All tests, analyses and measurements were conducted by individuals without knowledge of the identity or dosage level of the test substances, except for formulation preparation and analysis.

Rats were observed for viability at least twice daily during the dosage period. Observations for clinical signs were made daily before dosage administration. Body weights were recorded daily during the dosage period. Feed was monitored and replenished on an as-needed basis.

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a. See PROTOCOL DEVIATIONS, item 5.

Female rats were evaluated for the age of vaginal patency, beginning on PND 22. The appearance of a small "pin hole", a vaginal thread and complete vaginal opening (VO) were recorded on the days observed. Body weights were recorded on the day complete vaginal patency was observed; the day of complete opening was used in analysis. If a sufficient number of rats within any dosage group showed a persistent thread for greater than three days, then a separate analysis was conducted using the age at which the thread was first observed.

Estrous cycling was evaluated daily by examination of vaginal cytology beginning on the day vaginal patency was observed and continued until the day of sacrifice (postnatal day 42)[a]. Vaginal smears were classified as diestrus (presence of leukocytes and/or cornified/irregularly shaped nucleated epithelial cells), proestrus (presence of nucleated epithelial cells), or estrus (presence of cornified epithelial cells) and the stage was recorded daily. Age at first estrus was recorded.

2.7.6. Gross Necropsy

2.7.6.1. P Generation Rats

On DL 21, rats with litters assigned to study were sacrificed via carbon dioxide asphyxiation and discarded without further evaluation. The rat that did not deliver a litter by DG 23 was sacrificed and discarded without further evaluation.

2.7.6.2. F1 Generation Pups

Pups were sacrificed by an intraperitoneal injection of sodium pentobarbital (pups ≤ 14 days of age) or by carbon dioxide asphyxiation (pups ≥ 15 days of age) and discarded without further evaluation.

Pups that showed signs of being unthrifty or runted were sacrificed by an intraperitoneal injection of sodium pentobarbital on PND 3 or by carbon dioxide asphyxiation on PND 21 and discarded without further evaluation.

2.7.6.3. F1 Generation Rats

Gross lesions were retained in neutral buffered 10% formalin and examined histologically[b]. Unless specifically cited below, all other tissues were discarded. Representative photographs of gross lesions are available in the raw data.

On PND 42, F1 generation rats were anesthetized with exposure to carbon dioxide for no more than one minute and then sacrificed, necropsied and examined for gross lesions. Rats were sacrificed by decapitation beginning 2 hours after dosage administration and necropsies were completed by 1300 hours.

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a. See PROTOCOL DEVIATIONS, item 6.
b. See PROTOCOL DEVIATIONS, item 7 and 8.

A gross necropsy of the thoracic, abdominal and pelvic viscera was performed. Tissue trimming and histopathology was performed under the supervision of or by a Board Certified Veterinary Pathologist.

Ovaries without oviducts (paired), uterus, liver, kidneys (paired), pituitary and adrenal gland (paired) were individually weighed (to the nearest 0.1 mg) for all F1 generation rats assigned to study[a]. The uterus (without ovaries) was carefully dissected and trimmed of fascia and fat to avoid loss of luminal contents. The vagina was removed from the uterus at the level of the uterine cervix. The uterus and ovaries were fixed in Bouin's solution for 24 hours before being rinsed and retained in 70% alcohol until embedded in paraffin[b]. The thyroid with attached section of the trachea was fixed in neutral buffered 10% formalin for 24 hours. The thyroid was then dissected from the trachea, blotted dry, weighed (to nearest 0.1 mg) and retained 70% alcohol until embedded in paraffin. Histological examinations were performed to evaluate pathologic abnormalities and potential treatment-related affects of the test substances on the thyroid, ovaries and uterus of all F1 generation female rats.

Histological examinations were performed to evaluate pathologic abnormalities and potential treatment-related affects of the test substances on the thyroid, ovaries and uterus of all F1 generation female rats. Thyroid sections were stained with hematoxylin and eosin (H & E) and subjectively evaluated for follicular epithelial height and colloid area using a five point grading scale (1 = shortest/smallest; 5 = tallest/largest) and any abnormalities/lesions were noted. A minimum of two sections per thyroid were evaluated. Ovarian histology following H & E staining included evaluation of follicular development (including presence/absence of tertiary/antral follicles, presence/absence of corpora lutea, changes in corpus luteum development, changes in number of both primary and altretic follicles) in addition to any abnormalities/lesions, such as ovarian atrophy. Uterine histology documented incidences of uterine hyper- or hypotrophy as characterized by changes in uterine horn diameter and myometrial, stroma or endometrial gland development. Summaries of the histological findings with photomicrographs of significant observations are available in APPENDIX 11.

2.7.6.4. Hormone Analysis

Blood samples (at least 2 mL) for evaluation of thyroid hormones were collected from trunk blood immediately following sacrifice. The time of sample collection was documented in the raw data. Blood was collected and immediately placed into serum separator tubes to yield approximately 1000 mcL of serum, which was aliquotted into two vials of approximately 500 mcL each. One vial (500 mcL) was used for evaluating thyroid stimulating hormone (TSH) and the second vial (500 mcL) was used for evaluating thyroxine (T4). Serum samples were immediately frozen on dry ice and maintained frozen (-68°C to -78°C) until analysis (T_4) or shipment for analysis (TSH).

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a. See PROTOCOL DEVIATIONS, item 9.
b. See PROTOCOL DEVIATIONS, item 10.

Hormone analysis (T4) was conducted at the Testing Facility utilizing enzyme-linked immunosorbent (ELISA) procedures.

T4 analysis was conducted using commercially available Total Thyroxine (T4) Enzyme Linked Immunosorbant Assay (ELISA) kits. The kits were purchased from American Laboratory Products Company (Catalog # 025-BC-1007). 96 well plates provided in the kits were pre-coated with Sheep-anti-T4 antibodies.

Samples for T4 analysis were received according to the Testing Facility SOP and were equilibrated to room temperature prior to running the assays. 25 mcL of each standard or sample was added, in duplicate, to the appropriate wells. 100 mcL of working conjugate reagent was added to each well except for the blank and non-specific binding (NSB) wells. Plate was gently mixed for 30 seconds and then incubated for 60 minutes at room temperature. All wells were washed 5 times with 250 mcL of distilled water and the plate was tapped on absorbent paper to remove residual wash solution. 100 mcL of TMB reagent was added to each well. The plate was mixed gently for 10 seconds and covered then incubated at room temperature in the dark for 20 minutes. The reaction was stopped by adding 100 mcL of 1N HCl to each well. The plate was mixed gently for 30 seconds to ensure completion of color change from blue to yellow. The plate was then read on the Molecular Devices SpectraMax 190 at 450nm. Data were collected and T4 values calculated using SOFTMax Pro 4.0. External QC reference standards at 6.0 and 20 mcg/dL were within ±25% of the reference point; this was considered part of the acceptance criteria for the standard curves. The validated range (as established by the upper and lower standards) was between 2 and 25 mcg/dL. The intra-assay variation for the 6.0 mcg/dL T_4 reference standard was 7.14%, and the intra-assay variation for the 20.0 mcg/dL T_4 reference standard was 9.12%. Intra-assay variability tables are available in the raw data.

TSH analysis was conducted at CTBR Bio-Research Inc., Senneville, Quebec, Canada, utilizing Radioimmune Assay (RIA) procedures. External QC reference standards at 4.51, 7.50 and 11.48 ng/mL were within ±25% of the reference point; this was considered part of the acceptance criteria for the standard curves. The validated range (as established by the upper and lower standards) was between 0.85 and 31.00 ng/mL. The intra-assay variability were 11.2%, 9.4% and 5.9% for the 4.51, 7.50 and 11.48ng/mL TSH reference standards, respectively. Results of this analysis are available in APPENDIX 12.

2.7.7. Data Collection and Statistical Analyses

Data generated during the course of this study were recorded either by hand or using the *Argus Automated Data Collection and Management System*, the *Vivarium Temperature and Relative Humidity Monitoring System* and the *SOFTMaxPro* 4.0. All data were tabulated, summarized and/or statistically analyzed using the *Argus Automated Data Collection and Management System*, the *Vivarium Temperature and Relative Humidity*

Monitoring System, *Microsoft® Excel* (part of Microsoft® Office 97/2000/XP), Quattro Pro 8 and/or *The SAS System* (version 6.12).

2.7.8. Statistical Analyses

All data (weaning body weight, body weight gain from PND 22 to 42, age and body weight at vaginal opening, body and organ weights at necropsy, serum hormones and histology[a]) were analyzed by Analysis of Variance (ANOVA)[7]. Organ weights, age and body weight at vaginal opening were also analyzed by Analysis of Covariance (ANCOVA)[8] using the body weight at PND 21 as the covariate.

When statistically significant effects were observed ($p<0.05$, F/t statistic), treatment means were examined further using appropriate multiple comparison tests to compare the control with each treatment group [e.g., Dunnett's test[9] or LSMeans[10] (for ANCOVA)]. The specific *post hoc* test run is given on the tables.

In addition, data were evaluated for homogeneity of variance by Bartlett's test[11] for the ANOVA or by Levene's test[12] for the ANCOVA. There were no cases where heterogeneity of variance was evident, requiring the data to be transformed or analyzed using an appropriate nonparametric test (e.g., Kruskal-Wallis Nonparametric test)[13].

Les Freshwater of BioStat Consultants, Inc., Portage, Michigan, USA performed the statistical analyses to calculate the LSMeans in order to perform the ANCOVA and the Grubbs test for identification of statistical outliers (APPENDIX 13). Biological outliers and procedural errors were removed by the Study Director and are listed in APPENDIX 14.

a. See PROTOCOL DEVIATIONS, item 11

3. RESULTS

3.1. Mortality and Clinical Observations (Individual Data - APPENDIX 1)

All rats in all treatment groups survived until scheduled sacrifice. Excess salivation (slight to extreme) was observed in two female rats in the 25 mg/kg/day and five female rats in the 100 mg/kg/day 2-chloronitrobenzene dosage groups; and clear perinasal substance was observed in two female rats in the 100 mg/kg/day 2-chloronitrobenzene dosage group. There were no other clinical observations that were considered treatment related in the methoxychlor, DE-71, or 2-chloronitrobenzene dosage groups.

3.2. Body Weights (Figures 1 through 3; Summary - Table 1; Individual Data - APPENDIX 3)

Body weights of the juvenile female rats on postnatal days (PND) 22 through 42 and body weight gain PNDs 22 through 42, were unaffected by dosages of 12.5 or 50 mg/kg/day methoxychlor, 30 or 60 mg/kg/day DE-71, or 25 or 100 mg/kg/day 2-chloronitrobenzene. There were no biological or statistical differences across groups on any day.

3.3. Terminal Body Weights, Body Weight Gains and Organ Weights (Summaries - Tables 2 and 3; Individual Data - APPENDIX 4)

3.3.1. Corn Oil

Mean terminal body weights for the female rats in the corn oil dosage group were 170.7 g, with a coefficient of variation of 10.0%. The initial mean body weight of these rats was 57.9 g (coefficient of variation of 10.6%), resulting in a body weight gain of 112.7 g (coefficient of variation of 12.4%) over the course of the study.

Mean absolute weight of the liver, the only organ collected that demonstrated a significant weight change when compared to dose groups, was 8.4906 g, with a coefficient of variation of 16.3% for the female rats in the corn oil dosage group.

3.3.2. Methoxychlor

Terminal body weights for the female rats were unaffected by dosages of 12.5 or 50 mg/kg/day methoxychlor. There were no biological or statistical differences across groups at sacrifice.

The absolute weights of the pituitary, liver, kidneys (paired), adrenals (paired), thyroid (fixed), ovaries (paired), uterus (with fluid), and uterus (without fluid) were unaffected by dosages of 12.5 or 50 mg/kg/day methoxychlor. There were no biological or statistical differences across the 12.5 or 50 mg/kg/day dosage groups.

3.3.3. DE-71

Terminal body weights for the female rats were unaffected by dosages of 30 or 60 mg/kg/day DE-71. There were no biological of statistical differences across the 30 or 60 mg/kg/day dosage groups at sacrifice.

The absolute mean weight of the liver (10.6722g and 12.0915 g, respectively) was significantly increased ($p \leq 0.01$) in the 30 and 60 mg/kg/day dosage groups of DE-71. When the liver weights were analyzed by Analysis of Covariance (ANCOVA) using the PND 21 body weight as the covariate, the weight of the liver was significantly increased ($p \leq 0.01$) in both the 30 and 60 mg/kg/day dosage groups of DE-71.

The absolute weights of the pituitary, kidneys (paired), adrenals (paired), thyroid (fixed), ovaries (paired), uterus (with fluid), and uterus (without fluid) were unaffected by dosages of 30 or 60 mg/kg/day DE-71. There were no biological or statistical differences across the 30 or 60 mg/kg/day dosage groups.

3.3.4. 2-Chloronitrobenzene

Terminal body weights for the female rats were unaffected by dosages of 25 or 100 mg/kg/day 2-chloronitrobenzene. There were no biological of statistical differences across the 25 or 100 mg/kg/day dosage groups at sacrifice.

The absolute mean weights of the livers (10.9712 g and 13.4835 g, respectively) were significantly increased ($p \leq 0.01$) in the 25 and 100 mg/kg/day dosage groups of 2-chloronitrobenzene, when the liver weights were analyzed by the analysis of Variance (ANOVA) and by the ANCOVA using the PND 21 body weight as the covariate.

The adjusted mean weight of the uterus (without fluid) was significantly decreased ($p \leq 0.05$) in the 100 mg/kg/day dosage group of 2-chloronitrobenzene, when the uterus (without fluid) was analyzed by the analysis of Variance (ANOVA) and by the ANCOVA using the PND 21 body weight as the covariate.

The absolute weights of the pituitary, kidneys (paired), adrenals (paired), thyroid (fixed), ovaries (paired), and uterus (with fluid) were unaffected by dosages of 25 or 100 mg/kg/day 2-chloronitrobenzene.

3.4. Sexual Maturation (Summary - Table 3; Individual Data - APPENDIX 5)

3.4.1. Corn Oil

The average age of vaginal opening for the female rats in the corn oil dosage group was 31.467 days, with a coefficient of variation of 5.1%.

The average body weight at time of vaginal opening for the female rats in the corn oil dosage group was 110.013 g, with a coefficient of variation of 11.7%.

3.4.2. Methoxychlor

Both the average age of vaginal opening and average body weight at time of vaginal opening were significantly decreased ($p \leq 0.01$) for the female rats in the 50 mg/kg/day methoxychlor dosage group (27.533 days and 86.113 g, respectively), when the endpoints were analyzed by an ANOVA and by an ANCOVA using the PND 21 body weight as the covariate.

The average age of vaginal opening and the average body weight at time of vaginal opening for the female rats in the 12.5 mg/kg/day methoxychlor dosage group, was comparable to the control group.

3.4.3. DE-71

The average age of vaginal opening and the average body weight at time of vaginal opening was unaffected by dosages of 30 or 60 mg/kg/day DE-71 and the values were comparable to the control group.

3.4.4. 2-Chloronitrobenzene

The average age of vaginal opening and the average body weight at time of vaginal opening were significantly increased ($p \leq 0.01$) for the female rats in the 100 mg/kg/day 2-chloronitrobenzene dosage group (34.800 days and 127.507 g, respectively), compared to the control group.

The average age of vaginal opening and the average body weight at time of vaginal opening for the female rats in the 25 mg/kg/day 2-chloronitrobenzene dosage group, was comparable to the control group.

3.5. Gross Necropsy (Individual Data - APPENDIX 2)

There were no gross lesions in the corn oil dosage group rats. The left kidney of one female rat (144) in the 50 mg/kg/day methoxychlor dosage group was observed with a clear fluid filled cyst. Slight dilation of the right kidney was observed in one female rat (169) in the 60 mg/kg/day DE-71 dosage group. A black spleen was observed in 13 female rats and one female rat (194) with a secondary spleen attached was observed in the 100 mg/kg/day 2-chloronitrobenzene dosage group. There were no other necropsy observations that were considered treatment related in the 12.5 and 50 mg/kg/day methoxychlor, 30 and 60 mg/kg/day DE-71, or 25 mg/kg/day 2-chloronitrobenzene dosage groups.

3.6. Hormone Analyses (Summary - Table 4; Individual Data - APPENDIX 6)

3.6.1. Corn Oil

Serum thyroxine (T_4) levels were 8.031 mcg/dL, with a coefficient of variation of 16.8% and serum TSH levels were 3.606 ng/mL, with a coefficient of variation of 33.1%.

3.6.2. Methoxychlor

Serum T_4 and TSH levels were unaffected by dosages of 12.5 or 50 mg/kg/day methoxychlor.

3.6.3. DE-71

Serum T_4 levels were significantly reduced ($p \leq 0.01$) by dosages of 30 and 60 mg/kg/day of DE-71. Serum TSH levels were unaffected by dosages of 30 or 60 mg/kg/day DE-71.

3.6.4. 2-Chloronitrobenzene

Serum T_4 and TSH levels were unaffected by dosages of 25 or 100 mg/kg/day 2-chloronitrobenzene.

3.7. Histopathology (APPENDIX 11)

Thyroids were examined microscopically and the slides were coded, making the pathologist blind to the chemical and dose group. Subjective evaluated for follicular epithelial height and colloid area used a five point grading scale (1 = shortest/smallest; to 5 = tallest/largest). Ovaries were evaluated for the presence or absence of primary follicles, developing follicles, atretic follicles and corpora lutea, as well as for any histopathological finding that might suggest ovarian atrophy. Uteri were examined for cellular evidence of uterine pathology and the uterine diameter of each horn was morph-metrically measured.

3.7.1. Corn Oil

The control rats were the least affected concerning follicular epithelial cell height and the amount of colloid within follicles. One rat had a mild cyst in one ovary.

3.7.2. Methoxychlor

The rats that received 12.5 mg/kg/day of the test substance showed little effect on the thyroid; however, all rats that received 50 mg/kg/day had increased follicular epithelial cell height and slightly less colloid in the follicles. There were no signs of ovarian atrophy in any of the rats evaluated. The uteri of rats treated with methoxychlor showed

no evidence of uterine pathology and diameter measurements of the uterine horns revealed no meaningful differences across the 12.5 or 50 mg/kg/day groups.

3.7.3. DE-71

The rats that received 30 mg/kg/day of the test substance showed an increased follicular epithelial cell height and marginally less colloid in the follicles. The rats that received 60 mg/kg/day of the test substance showed the greatest affect on the thyroid; all fifteen thyroids were moderately increased follicular epithelial cell height and colloid depletion. There were no signs of ovarian atrophy in any of the rats evaluated. The uteri of rats treated with DE-71 showed no evidence of uterine pathology and diameter measurements of the uterine horns revealed no meaningful differences across the 30 or 60 mg/kg/day groups.

3.7.4. 2-Chloronitrobenzene

The rats that received 25 or 100 mg/kg/day of the test substance showed an increased follicular epithelial cell height and marginal colloid depletion in the follicles; only three rats in the 25 mg/kg/day and one rat in the 100 mg/kg/day dosage groups had normal thyroid histopathology. There were no signs of ovarian atrophy in any of the rats evaluated. The uteri of rats treated with 2-chloronitrobenzene showed no evidence of uterine pathology and diameter measurements of the uterine horns revealed no meaningful differences across the 25 or 100 mg/kg/day groups.

4. DISCUSSION

This study was done as step toward validating the Pubertal Female Assay. The transferability of the protocol was evaluated using methoxychlor, DE-71 and 2-chloronitrobenzene, with the first two chemical compounds known to affect the endocrine system through different pathways and/or mechanisms of action. This study is expected to provide a means of screening the effects of potential endocrine disruptors that may alter a number of endocrine-dependent mechanisms, including estrogenic-, androgenic-, and thyrotropic-like processes [(14)]. The endpoints in this study were chosen to reflect specific changes in general toxicity (growth), age and weight at sexual maturation, vaginal cytology, reproductive organ and thyroid weights and histology (including colloid depletion and follicular cell height), non-reproductive organ weights (liver, kidney, pituitary, and adrenals) and systemic hormone concentrations (T_4 and TSH), in part, in response to methoxychlor, DE-71 or 2-chloronitrobenzene exposure.

There was little visible evidence of general toxicity in the juvenile female rats caused by 21 days of treatment by the three test chemicals. There were no deaths on the study and there were no significant differences in the mean body weights across the corn oil control group and the methoxychlor, DE-71 or 2-chloronitrobenzene treatment groups. There were no clinical or necropsy observations that were related to exposure to methoxychlor or DE-71; however, 2-chloronitrobenzene exposure caused a dose dependent increase in excess salivation and a black spleen was observed at necropsy in 86.7% of the females in

the 100 mg/kg/day dosage group. 2-Chloronitrobenzene is known to induce methemoglobin, which reduces red cell cycle time and increases red cell clearance, producing discolored and enlarged spleens (NTP 1997). Liver weights were significantly increased by DE-71 and 2-chloronitrobenzene exposure; organ weights were unaffected by treatment of methoxychlor. Induction of hepatic microsomal phase II enzymes and subsequent enlargement of the liver are known to occur with DE-71 and 2-chloronitrobenzene exposure[15, 16].

Two of the chemical exposures did affect sexual maturation. The average age of vaginal opening and average body weight at time of vaginal opening were significantly decreased by methoxychlor and significantly increased by 2-chloronitrobenzene dosage. The average age of vaginal opening and average body weight at time of vaginal opening were unaffected by DE-71. This is in contrast with the findings of Stoker et al [17] that observed a significant delay in the age of vaginal opening in Wistar rats that were treated with 60 mg/kg/day DE-71 for 21 days[17]. Increased follicular epithelial cell height and colloid depletion was evident in the thyroids from methoxychlor, DE-71 or 2-chloronitrobenzene exposure, indicating the rats to be in a hypothyroid state. Ovarian and uterine histopathology was normal for all three test chemicals.

5. CONCLUSION

The three test chemicals and their target/mechanism of action are as follows: (1) methoxychlor - a xeno-estrogen through the α-estrogen receptor, an anti-estrogen through the β -estrogen receptor, and an anti-androgen through an androgen receptor-mediated mechanism; (2) DE-71, a commercial mixture of polybrominated diphenyl ethers - a thyroid active chemical that increases clearance of thyroxine (T_4) through induction of hepatic microsomal phase II enzyme uridine diphospho-glucuronosyl transferase (UDPGT) activity; and (3) 2-chloronitrobenzene - a nitroaromatic that induces methemoglobinemia but is expected to have no endocrine effects[18].

Methoxychlor is a pesticide with estrogenic properties in the DDT family. Rat studies show that exposure to methoxychlor in food or water harms the ovaries, uterus, and mating cycle in females, and the testes and prostate in males. Fertility is decreased in both male and female rats [19]. In the current study, 50 mg/kg/day methoxychlor exposure caused the average age of vaginal opening and average body weight at time of vaginal opening to be significantly decreased and thyroid follicular epithelial cells to be increased in height and have colloid depletion. There was no effect observed on estrous cycling or ovarian pathology and there was no change in T_4 or TSH levels.

DE-71 is a brominated diphenyl used as a flame retardant in rigid and flexible polyurethane foam, epoxides, laminates, adhesives, and coatings. A similarly designed study conducted by EPA found DE-71 an similar dose levels to cause decreased serum T_4 levels, thyroid changes, increased liver weight, decreased seminal vesicle and ventral prostate weight, and delays in preputial separation [15]. In the current study, serum T_4 levels were significantly reduced and liver weights were significantly increased in the 30 and 60 mg/kg/day DE-71 dosage groups. A dosage-dependent increase in follicular

epithelial cell height and marginal colloid depletion was evident in the thyroids from the 30 and 60 mg/kg/day dosage levels indicating a hypothyroid state was reached but there was no effect on sexual maturation, estrous cycling or ovarian pathology.

2-Chloronitrobenzene is an intermediate in the manufacture of pesticides (parathion), pharmaceuticals (4-acetylaminophenol), dyes, lumber preservatives, and photographic chemicals. In a reproductive and fertility study in mice conducted by the National Toxicology Program (NTP), 2-chloronitrobenzene exposure caused increase in liver and spleen weights with no reproductive toxicity [16]. In the current study, a dose dependent increase in excess salivation was observed in the 25 and 100 mg/kg/day dosage groups and a black spleen was observed at necropsy in 86.7% of the females in the 100 mg/kg/day dosage group. Liver weights were significantly increased in the 25 and 100 mg/kg/day dosage groups. The average age of vaginal opening and average body weight at time of vaginal opening were significantly increased in the 100 mg/kg/day dosage group. Increased follicular epithelial cell height and colloid depletion was evident in the thyroids from the 25 and 100 mg/kg/day dosage groups, indicative of a hypothyroid state. Ovarian and uterine histopathology was normal and there was no change in T_4 or TSH levels.

3 JAN 06

Alan M. Hoberman, Ph.D., DABT Date
Director of Research

03 JAN 06

Raymond G. York, Ph.D., DABT Date
Associate Director of Research
and Study Director

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7. PROTOCOL DEVIATIONS

1. The storage condition of the vehicle (Lot AO-003) was not documented. This deviation did not adversely affect the outcome or interpretation of the study because the vehicle was stored according to the conditions specified in the protocol.

2. The peroxide content of the corn oil used to prepare the dose formulations was 3.15 mEq/mL, which was outside of the acceptance criteria listed in the protocol. This deviation did not adversely affect the outcome or interpretation of the study because the out of specification range was minimal.

3. The peroxide content of the corn oil used for preparing the formulation was reported in mEq/Kg rather than mEq/mL. This deviation did not adversely affect the outcome or interpretation of the study because this was a typographical error and had no impact on the scientific interpretation of the data.

4. A 1 g vehicle reserve sample was not taken of lots AO-001 or AO-002 prior to combining the lots to form lot AO-003. This deviation did not adversely affect the outcome or interpretation of the study because this did not result in the loss of any data.

5. On day 29 of observation (OD 29), 5 April 2005, rats 101 - 110, in the 0 mg/kg/day dosage group were dosed from 0654 to 0659. This deviation did not adversely affect the outcome or interpretation of the study because the time started (early) was very small and this was a single occurrence.

6. On 19 APR 05, OD 42, estrus evaluations were not performed on the following rats, on the day of sacrifice. See below.

Rat Number	Dosage group	Rat Number	Dosage group
101	I	137	III
102	I	146	IV
103	I	147	IV
104	I	148	IV
105	I	149	IV
106	I	150	IV
107	I	151	IV
116	II	152	IV
117	II	161	V
118	II	162	V
119	II	163	V
120	II	164	V
121	II	165	V
122	II	166	V
123	II	176	VI
124	II	177	VI
125	II	178	VI
126	II	179	VI
127	II	180	VI
131	III	181	VI
132	III	191	VII
133	III	192	VII
134	III	193	VII
135	III	194	VII
136	III	NA	NA

This deviation did not adversely affect the outcome or interpretation of the study because sufficient data were collected in order to evaluate this parameter and the missing data was across all dose groups.

7. All gross lesions were not histologically evaluated. Only gross lesions associated with the ovaries, uterus and thyroid were evaluated at the request of the Study Director. This deviation did not adversely affect the outcome or interpretation of the study because the purpose of the study was to validate the assay and not the toxicity of the test substances. Therefore sporadic gross lesions from non-target organs did not have to be evaluated.

8. The left kidney (gross lesion) of rat 144, in Group III, was inadvertently lost and not saved in neutral buffered 10% formalin. This deviation did not adversely

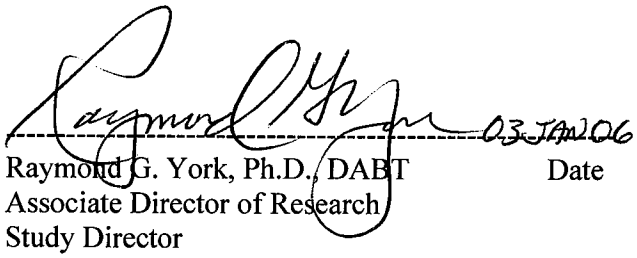
affect the outcome or interpretation of the study because it was a single occurrence.

9. The original weight recorded for paired ovaries for rat 129, in Group II was presumed incorrectly recorded and therefore a fixed weight was taken. This deviation did not adversely affect the outcome or interpretation of the study because sufficient data were available to evaluate this parameter.

10. The ovaries of rat 132 in Group III were not retained. This deviation did not adversely affect the outcome or interpretation of the study because sufficient data were available to evaluate this parameter.

11. The histology data was not analyzed by Analysis of Variance (ANOVA). This deviation did not adversely affect the outcome or interpretation of the study because this statistical analysis was deemed unnecessary.

All deviations are documented in the raw data.



03 JAN 06

Raymond G. York, Ph.D., DABT Date
Associate Director of Research
Study Director