FINAL REPORT

Study Title

Interlaboratory Validation of the Pubertal Male Assay

Sponsor's Work Assignment and Task Number: WA 4-15, Task 4

Author

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(Study Director)

Study Completed On

3 January 2006
(Final Report)

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Laboratory Project ID

CR-DDS Argus Division Protocol Number: RTP00002

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

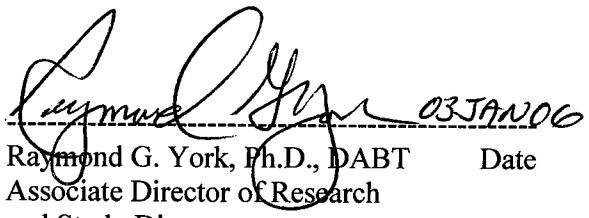
This study was conducted according to U.S. Environmental Protection Agency. Federal Insecticide, Fungicide and Rodenticide Act/Toxic Substances Control Act (FIFRA/TSCA); Good Laboratory Practice Standards; Final Rule. 40 CFR Part 160/Part 792 with the exception that the statistical analysis conducted 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 MALE ASSAY

CR-DDS ARGUS DIVISION PROTOCOL NUMBER: RTP00002

SPONSOR'S WORK ASSIGNMENT AND TASK NUMBER: WA 4-15, Task 4

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ABSTRACT

This study was done as a step toward validating the Male Pubertal Assay as an alternative assay in the Tier I battery. The transferability of the protocol was evaluated using vinclozolin, DE-71, 2-chloronitrobenzene and dibutyl phthalate, three of the chemical compounds were 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 male rats, 15 per group, were dosed between 0700 and 0900 hours each day for 31 days via oral gavage with corn oil (vehicle control), vinclozolin at 30 and 100 mg/kg/day, DE-71 at 30 and 60 mg/kg/day, 2-chloronitrobenzene at 25 and 100 mg/kg/day and dibutyl phthalate at 500 and 1000 mg/kg/day. Dosages were formulated using corn oil as the vehicle and administered at 2.5 mL/kg.

Viabilities were recorded twice daily. Clinical observations were recorded once daily during the predosage and dosage periods. Body weights were recorded on postnatal days 0, 4, 7, 14 and 21 (PNDs 0, 4, 7, 14 and 21) and daily postweaning (PNDs 22 through 53). Male rats were evaluated for age and body weight at preputial separation, beginning on PND 30. Necropsies were performed on PND 53, beginning two hours after dose administration and prior to 1300 hours. Trunk blood was collected from anesthetized rats following decapitation for testosterone, thyroxine (T_4) and thyroid stimulating hormone (TSH) analyses. Testes, epididymides, ventral prostate, thyroid, dorsolateral prostate, seminal vesicles, levator ani with bulbocavernosus muscle, livers, kidneys, pituitaries and adrenal glands were collected and weighed and histopathology was conducted on the thyroids, right testis and right epididymis of all F1 generation male rats.

- **Vinclozolin**

There were no deaths on the study; no clinical or necropsy observations were considered treatment related in rats treated with 30 or 100 mg/kg/day of vinclozolin. Body weights were significantly lower relative to controls on PNDs 51, 52 and 53, by dosages of 100 mg/kg/day. The weight of the left testis and adrenals (paired) were significantly increased and the weight of the seminal vesicles, epididymides (paired) and levator ani were significantly reduced in the 100 mg/kg/day dosage group. Both the average age of preputial separation and average body weight at time of preputial separation were

significantly increased for the rats in the 30 and 100 mg/kg/day dosage groups. Serum T_4 levels were significantly reduced in the 30 and 100 mg/kg/day dosage groups. Serum testosterone levels and serum TSH levels were unaffected by dosages of 30 and 100 mg/kg/day. Treatment of rats with 30 and 100 mg/kg/day resulted in no effects on the thyroid, and there was no significant evidence of testicular or epididymal atrophy.

- **DE-71**

There were no deaths on the study; no clinical or necropsy observations were considered treatment related in rats treated with 30 or 60 mg/kg/day of DE-71. Body weights were unaffected by dosages up to 60 mg/kg/day. The mean absolute weights of the liver were significantly increased in the 30 and 60 mg/kg/day dosage groups. The average age of preputial separation was significantly increased but not the average body weight at time of preputial separation in the 60 mg/kg/day dosage group. Serum T_4 levels were significantly reduced in the 30 and 60 mg/kg/day dosage groups. Serum testosterone levels and serum TSH levels were unaffected by dosages of 30 and 60 mg/kg/day. Treatment of the rats with 30 mg/kg/day resulted in no effects on the thyroid, and those treated with 60 mg/kg/day had minor thyroid effects. There was no significant evidence of testicular or epididymal atrophy.

- **2-Chloronitrobenzene**

There were no deaths on the study. Urine-stained abdominal fur was observed in four rats, excess salivation was observed in two rats and clear perinasal substance was observed in one rat treated with 100 mg/kg/day of 2-chloronitrobenzene. A dark red spleen was observed in one and two rats in the 25 and 100 mg/kg/day dosage groups, respectively. A black spleen was observed in two rats and an enlarged black spleen was observed in seven rats in the 100 mg/kg/day dosage group. Body weights were significantly reduced on PNDs 51, 52 and 53 by dosages of 100 mg/kg/day. Liver and kidney weights were significantly increased in the 25 and 100 mg/kg/day dosage groups. The weights of the right testis, ventral prostate and levator ani were significantly reduced in the 100 mg/kg/day dosage group. The average age of preputial separation was significantly increased in the 100 mg/kg/day dosage group. Serum T_4 levels were significantly increased in the 25 and 100 mg/kg/day dosage groups. Serum testosterone levels and serum TSH levels were unaffected by dosages of 25 and 100 mg/kg/day. Significant testicular changes, degeneration of the germinal epithelium and the presence of intralumenal multinucleated giant cells, occurred in the 100 mg/kg/day dosage group.

- **Dibutyl phthalate**

There were no deaths on the study. Excess salivation was observed in three rats treated with 500 mg/kg/day and in five rats treated with 1000 mg/kg/day of dibutyl phthalate. Clear perinasal substance was observed in two rats treated with 1000 mg/kg/day of dibutyl phthalate. Necropsy examination revealed the testes, epididymides and seminal vesicles were small in rats treated with 500 and 1000 mg/kg/day of dibutyl phthalate. Body weights were significantly reduced on PNDs 45 and 47 through 53 by dosages of

1000 mg/kg/day. The weights of the testes, epididymides and levator ani were significantly reduced in the 500 and 1000 mg/kg/day dosage groups. The weights of the seminal vesicles were significantly reduced in the 500 and 1000 mg/kg/day dosage group and the weight of the liver was significantly increased in the 1000 mg/kg/day dosage group. The average age of preputial separation was significantly increased in the 500 and 1000 mg/kg/day dosage groups. Serum T_4 levels were significantly decreased in the 500 and 1000 mg/kg/day dosage groups. Serum testosterone levels and serum TSH levels were unaffected by dosages of 500 and 1000 mg/kg/day. Treatment of the rats with 500 or 1000 mg/kg/day resulted in minor thyroid effects. Treatment of the rats with 500 or 1000 mg/kg/day resulted in minor increases in follicular epithelial cell height and marginal colloid depletion in the follicles. Significant testicular changes, minimal to marked degeneration of the germinal epithelium of the right testis and/or the presence of intralumenal multinucleated giant cells, occurred in rats treated with 500 and 1000 mg/kg/day of dibutyl phthalate. Minimal to marked spermatic depletion, minimal to mild lymphocytic infiltration and minimal to moderate sloughing of the epithelium in the lumen of the right epididymis was observed in rats treated with 500 and 1000 mg/kg/day of dibutyl phthalate. Significant testicular changes where seminiferous tubules were lined only with Sertoli cells occurred in the 500 and 1000 mg/kg/day dosage groups.

1. OBJECTIVES

The purpose of this project was to quantify the effects of chemicals on pubertal development and thyroid function in the intact juvenile/peripubertal male rat. This assay detects chemicals that display anti-thyroid, estrogenic, or androgenic/anti-androgenic activity.

The EPA has selected four test chemicals for evaluation in the male pubertal assay, and has selected the low and high target doses (in mg/kg/day) for each of them. The four test chemicals and their target/mechanism of action are as follows: (1) vinclozolin - an anti-androgen through competitive binding of metabolites M1 and M2 to the androgen receptor, M1 binds weakly to the rat progesterone receptor; (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; (3) 2-chloronitrobenzene - a nitro-aromatic, inducing methemoglobinemia and which has been shown to decrease sperm motility in mice; and (4) dibutyl phthalate - an anti-androgen through the androgen or β-estrogen receptor.

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 Discovery and Development Services, Argus Division, 905 Sheehy Drive, Building A, Horsham, Pennsylvania 19044-1241, USA

2.1.3. Study Number

RTP00002

2.1.4. Sponsor's Work Assignment and Task Number

WA 4-15, 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 intact juvenile/peripubertal male rat. This assay detects chemicals that display anti-thyroid, estrogenic or androgenic/anti-androgenic activity [androgen receptor (AR) 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

The study was conducted in compliance with Good Laboratory Practice (GLP) regulations of the EPA[(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.
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 Sr., B.S. (Director of Operations, Argus Division)
Gerard M. Zimmerman, ALAT (Study Supervisor, Argus Division)
Daniel E. Fisher, (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 and testosterone analyses
Melissa A. Snyder, B.S. (Formulation Laboratory Technician, Argus Division) - Thyroxine and testosterone analyses
Richard Norlin, M.S. (Principal Investigator, Worcester Division, Worcester, Massachusetts, USA) - Formulation Analyses
Jerry L. Quance, D.V.M., DACVP (Principal Investigator, Pathology Associates Division, Frederick, Maryland, USA) - Histopathology Evaluations

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 and Scientist, Immunochemistry, Laboratory Sciences, 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)
Cheryl L. Karvounis, A.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

2.1.19.1. P Generation Rats

P Generation Rat Arrival	22 FEB 05
Delivery Period (PND[a] 0)	07 MAR 05 - 09 MAR 05
PND 4 Sacrifice	11 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 23 through PND 53)	31 MAR 05 - 01 MAY 05
Sexual Maturation (Beginning on PND 30 - Preputial Separation) Evaluation	07 APR 05 - 30 APR 05
PND 53 Sacrifice (Scheduled sacrifice F1 generation rats)	30 APR 05 - 01 MAY 05

2.1.20. Records Maintained

The original report, raw data and reserve samples of the bulk test substances and 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 the Testing Facility following issue of the final report. Disposition of the remaining bulk test substances will be documented in the raw data.

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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.

2.2. Test Substances 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
Vinclozolin (50471-44-8)	White powder	15 DEC 04	Room temperature	329-72B	OCT 07
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
Dibutyl Phthalate (84-74-2)	Colorless liquid	16 DEC 04	Room temperature	00323PU[a]	11 OCT 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. 415-01 and provided by the Sponsor to the Testing Facility. The results from these analyses are available in APPENDIX 9.

a. See PROTOCOL DEVIATIONS, item 1.

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 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 formulations was analyzed to make certain that the peroxide content was <3 mEq/mL[c].

2.4. Test Substance Preparation and Storage Conditions

Solutions/suspensions for administration were prepared once at the Testing Facility. Prepared solutions/suspensions 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. At the Sponsor's request, the corn oil from lots AO-001 and AO-002 were combined in a 50 L carboy and thoroughly mixed. As a result, the combined lot of corn oil was assigned lot number of AO-003 by the Testing Facility.
b. See PROTOCOL DEVIATIONS, item 2.
c. See PROTOCOL DEVIATIONS, items 3 and 4.

2.4.1. Sample Information

Sample Type	Size	Date Retained	Storage Conditions	Shipped To/Shipping Conditions	Date Shipped
Vinclozolin					
Bulk Test Substance[a]	1 g	01 MAY 05	Room temperature	Sponsor[b]/Ambient conditions	02 MAY 05
Homogeneity[c] 12 mg/mL 40 mg/mL	1 mL	 10 MAR 05 11 MAR 05 24 MAR 05	Refrigerated	CR-DDS Worcester Division[d]/On cold packs	 11 MAR 05 11 MAR 05 24 MAR 05
Concentration[e] 12 mg/mL 40 mg/mL	1 mL	 10 MAR 05 21 MAR 05 11 MAR 05 22 MAR 05	Refrigerated	CR-DDS Worcester Division[d]/On cold packs	 11 MAR 05 22 MAR 05 11 MAR 05 22 MAR 05
Bulk Test Substance Reserve[f]	0.56 g	11 MAY 05	Room temperature	Testing Facility Archives	17 MAY 05
DE-71					
Bulk Test Substance[a]	1 g	01 MAY 05	Room temperature	Sponsor[b]/Ambient conditions	02 MAY 05
Concentration[g] (all levels)	1 mL	18 MAR 05 21 MAR 05	Refrigerated	CR-DDS Worcester Division[d]/On cold packs	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	01 MAY 05	Room temperature	Sponsor[b]/Ambient conditions	02 MAY 05
Concentration[h] (all levels)	1 mL	10 MAR 05 21 MAR 05	Refrigerated	CR-DDS Worcester Division[d]/On cold packs	11 MAR 05 22 MAR 05
Bulk Test Substance Reserve	1 g	11 MAY 05	Room temperature	Testing Facility Archives	17 MAY 05

a. A sample of each bulk test substance was taken on the last day of treatment and shipped for analysis.
b. Battelle Marine Sciences Laboratory, Sequim, Washington, USA.
c. Homogeneity analysis of the prepared formulations of vinclozolin was conducted during a prestudy preparation (12 and 40 mg/mL concentrations), as well as prior to the initiation of dosage (40 mg/mL concentration). Quadruplicate samples were taken from the top, middle and bottom of the vinclozolin formulations. Two samples from each quadruplicate 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. Six samples were taken from each preparation on the day prepared from the Vinclozolin 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.
f. See PROTOCOL DEVIATIONS, item 5.
g. 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.
h. 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.

Sample Type	Size	Date Retained	Storage Conditions	Shipped To/Shipping Conditions	Date Shipped
Dibutyl phthalate					
Bulk Test Substance[a]	1 g	01 MAY 05	Room temperature	Sponsor[b]/Ambient conditions	02 MAY 05
Concentration[c] (all levels)	1 mL	10 MAR 05 21 MAR 05	Refrigerated	CR-DDS Worcester Division[d]/On cold packs	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[e]	1 g	11 MAY 05	Refrigerated	Testing Facility Archives	17 MAY 05

a. A sample of each 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 dibutyl phthalate 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. See PROTOCOL DEVIATIONS, item 6.

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 DE-71, 2-chloronitrobenzene and dibutyl phthalate 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 homogeneity analysis for the prepared formulations for vinclozolin, and concentration results for all test substances used on study are available in APPENDIX 9. The concentration results from the pre-study preparation of DE-71 and 2-chloronitrobenzene and the start of study concentration results for the 2-chloronitrobenzene are located in the Method Validation and Formulation Sample Analysis report for study RTP00001 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 male rats assigned to study were delivered at the Testing Facility.

2.5.4. Sex

Male (NOTE: P generation dams were provided by the Supplier to maintain the F1 generation pups and were not considered part of the Test System.)

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 Selected F1 Generation Male Rats	135
Dates of Birth	08 MAR 05 - 09 MAR 05
Weight (g) at Study Assignment	50.1 - 69.0

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 of ten pups per litter, maximizing the number of males left in the litter and retaining females 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, 135 F1 generation male 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 was selected for study by eliminating an equal number of pups from the heavy end and the light end of distribution, leaving the number of male rats needed for study in the middle. Male rats were assigned to treatment groups such that the mean body weights and variances for all groups were similar. Littermates were not assigned to the same dosage group.

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

On PND 21, male pups were temporarily tail-marked with a Sharpie® marker with the numbers 01, 02, etc., through the nth male in each litter. The pup's weights were then recorded into a computer file that identifies each male 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 male 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 pup weights on PND 21, 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 nine pups were allowed to be assigned to study from each litter, the lightest and/or heaviest of the 10th through nth male pups from those litters containing more than nine males were considered ineligible for study assignment; those pups were crossed off of the body weight-ordered list. The PND 21 body weight rankings for the day were then examined by the Study Director, who approved any further exclusion for body weight considerations.

A table of random units was then used to assign all eligible pups to dosage groups (Groups I through IX); the 9 lightest-weight pups were each randomly assigned to 1 of the 9 dosage groups, then the 9 next lightest-weight pups were assigned, and so on until all eligible pups were 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 tail-tattooed prior to weaning them into group housing in nesting boxes.

Pups were placed onto study on the basis of clinical observations and body weights recorded on PND 21. After assignment to treatment groups, rats were housed in groups of three rats per cage, such that each cage contained 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 (No. MSPT 20101, Gey Band and Tag Co., Inc., Norristown, Pennsylvania, USA) following natural delivery of the pups. Cage tags were marked with the study number, permanent rat number, sex, 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 (Spaulding Electric Tattoo Marker, Spaulding and Rogers Manufacturing, Inc., Voorheersville, New York, USA, with AIMS Black Pigment #242, AIMS, Inc., Piscataway, New Jersey, USA). Cage tags were marked with the study number, permanent rat number, sex, generation, test substance identification 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 male rats were group housed in nesting boxes (three pups per nesting box) by dosage group.

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

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

Cage racks were changed approximately every other week. Nesting boxes and bedding were 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.

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)	Concen-tration (mg/mL)	Dosage Volume (mL/kg)	Number of Rats	Assigned F1 Generation Rat Numbers
I	Corn Oil (I)	0	0	2.5	15	301 - 315
II	Vinclozolin (A)	30	12	2.5	15	316 - 330
III	Vinclozolin (B)	100	40	2.5	15	331 - 345
IV	DE-71 (C)	30	12	2.5	15	346 - 360
V	DE-71 (D)	60	24	2.5	15	361 - 375
VI	2-Chloronitrobenzene(E)	25	10	2.5	15	376 - 390
VII	2-Chloronitrobenzene(F)	100	40	2.5	15	391 - 405
VIII	Dibutyl Phthalate (G)	500	200	2.5	15	406 - 420
IX	Dibutyl Phthalate (H)	1000	400	2.5	15	421 - 435

a. Assigned Group Letter
b. The test substances were considered 100% active for the purpose of dosage calculations.

2.7.2. Rationale for Dosage Selection

Vinclozolin, dibutyl phthalate 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 generation male rats weighing < 50 ± 5 grams was a 22 gauge, stainless steel gavage needle, either 1 or 1.5 inches with a 1.25 mm ball tip. The needle size used for F1 generation male rats weighing > 50 ± 5 grams was a 20 gauge, stainless steel feeding needle, 1.5 inches with a 2 mm silicone ball tip. As of 26 April 2005, the needle size used for test substance administration were 16 gauge, stainless steel feeding needles, 2 or 3 inches with a 3 mm stainless ball tip.

2.7.4. Frequency of Administration

2.7.4.1. P Generation Rats

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

2.7.4.2. F1 Generation Pups/Rats

F1 generation rats were given the test substances and/or vehicle on PNDs 23 through 53. Dosages were given once daily, between 0700 hours and 0900 hours each day. 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

Thirty-two timed-mated female rats were received at the Testing Facility. Rats were observed for viability at least twice daily. Feed was monitored and replenished on an as-needed basis.

2.7.5.2. F1 Generation Pups

Day 0 of lactation [postnatal day 0 (PND 0)] was defined as the day of birth and was also the first day on which all pups in a litter were individually weighed (pup body weights were recorded after all pups in a litter were 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 DLs 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 postweaning period. Observations for clinical signs were made once daily during the predosage period and daily before dosage administration. Body weights were recorded daily during the postweaning period and on the day sacrificed.

Male rats were evaluated for the age of preputial separation beginning on PND 30. The appearance of complete preputial separation was recorded[a]. Body weights at complete preputial separation were recorded.

2.7.6. Gross Necropsy

2.7.6.1. P Generation Rats

Rats that did not deliver a litter by DG 23 were sacrificed via carbon dioxide asphyxiation and discarded without further evaluation.

Dams with litters not assigned to the study due to an insufficient number of pups were sacrificed via carbon dioxide asphyxiation and discarded without further evaluation.

On postpartum day 21, P generation dams with litters assigned to study were sacrificed via carbon dioxide asphyxiation and discarded without further evaluation.

2.7.6.2. F1 Generation Pups

All pups not selected for continued observation on PND 4 were sacrificed via intraperitoneal injection of sodium pentobarbital and discarded without further evaluation.

All pups not selected for continued observation on PND 21 were sacrificed via carbon dioxide asphyxiation 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.

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a. See PROTOCOL DEVIATIONS, item 7.
b. See PROTOCOL DEVIATIONS, items 8 and 9.

On PND 53, F1 generation male rats were anesthetized by exposure to carbon dioxide for no more than one minute and sacrificed by decapitation. Rats were sacrificed beginning 2 hours after dosage administration and necropsies were completed by 1300 hours.

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.

The testes (left and right), epididymides (paired), ventral prostate, dorsolateral prostate, seminal vesicles (with coagulating glands with and without fluid), levator ani plus bulbocavernosus muscles, liver, kidneys (paired), pituitary and adrenal glands (paired) were weighed (to the nearest 0.1 mg) for all F1 generation male rats assigned to study. Apparently small tissues and those containing fluid were weighed immediately after collection. The right testis and epididymis were fixed in Bouin's solution for 24 hours before being rinsed and retained in 70% alcohol until embedded in paraffin. The thyroid with attached section of trachea was fixed in neutral buffered 10% formalin for 24 hours. The thyroid was then dissected from the trachea, blotted dry, weighed and retained in 70% alcohol until embedded in paraffin.

Histological examinations were performed on the thyroid, right testis and epididymis of all F1 generation male 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. In addition to gross lesions such as atrophy or tumors, detailed testicular histopathological examination (following paraffin-embedding and transverse sectioning of 4 to 5 mcm thickness) was conducted to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells or sloughing of spermatogenic cells into the lumen. Examination of the intact epididymis included the caput, corpus and cauda (accomplished by evaluation of a longitudinal section) was conducted in order to identify such lesions as sperm granulomas, leukocytic infiltration (inflammation), aberrant cell types within the lumen, or the absence of clear cells in the cauda epididymal epithelium. Summaries of the histological findings with photomicrographs of significant observations are available in APPENDIX 11.

2.7.6.4. Hormone Analysis[a]

Blood samples (at least 2 mL) for evaluation of thyroid hormones and testosterone 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

a. See PROTOCOL DEVIATIONS, items 10 and 11.

used for evaluating thyroxine (T_4) and testosterone. Serum samples were immediately frozen on dry ice and maintained frozen (-68°C to -78°C) until analysis (T_4 and testosterone) or shipment for analysis (TSH).

Hormone analysis (T_4 and testosterone) was conducted at the Testing Facility.

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 20.0 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 18.85%, and the intra-assay variation for the 20.0 mcg/dL T_4 reference standard was 9.83%. Intra-assay variability tables are available in the raw data.

Testosterone analysis was conducted using commercially available Testosterone Enzyme Linked Immunosorbant Assay (ELISA) kits. The kits were purchased from Biomeda (Catalog # EU1048). 96 well plates provided in the kits were pre-coated with Anti-rabbit IgG antibodies.

Samples for testosterone analysis were received according to the Testing Facility SOP and were equilibrated to room temperature prior to running the assays. 10 mcL of each standard or sample was added, in duplicate, to the appropriate wells. 50 mcl of antibody solution was added to each well except the blank and NSB wells. 50 mcL of enzyme conjugate was added to each well except for the blanks. The plate was then incubated for 60 minutes at room temperature. All wells were washed 5 times with 250 mcL of wash buffer and the plate was tapped on absorbent paper to remove residual wash solution. 100 mcL of Solution A and 100 mcL of solution B were added to each well. The plate was covered and then incubated at room temperature for 30 minutes. The reaction was stopped by adding 50 mcL of 1N H_2SO_4 to each well. The plate was then read on the

Molecular Devices SpectraMax 190 at 450nm. Data were collected and Testosterone values calculated using SOFTMax Pro 4.0. External QC reference standards at 4.0 and 14 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 0.5 and 20.0 ng/mL. The intra-assay variation for the 4.0 ng/mL testosterone reference standard was 13.97%, and the intra-assay variation for the 20.0 ng/mL testosterone reference standard was 6.96%. Intra-assay variability tables are available in the raw data.

TSH analysis was conducted at CTBR Bio-Research Inc., Senneville, Quebec, Canada, utilizing radioimmunoassay (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 11.5%, 6.5% and 17.5% for the 4.51, 7.50 and 11.48 ng/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. Statistics

All data (weaning body weight, body weight gain from PND 23 to 53, age and body weight at preputial separation, 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 preputial separation 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 the Bartlett's test[(11)] for the ANOVA or by Levene's test[(12)] for the ANCOVA. There were no cases where

a. See PROTOCOL DEVIATIONS, items 12

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.

3. RESULTS

3.1. Clinical Observations (Individual Data - APPENDIX 1)

All rats in all treatment groups survived until scheduled sacrifice.

3.1.1. Corn Oil

There were no clinical observations in rats treated with corn oil.

3.1.2. Vinclozolin

One rat treated with 100 mg/kg/day of vinclozolin was observed with slight excess salivation on a single occasion during the study. This observation was not considered treatment related as it was a single occurrence in only one rat. There were no other clinical observations in rats treated with 30 or 100 mg/kg/day of vinclozolin.

3.1.3. DE-71

There were no clinical observations in rats treated with 30 or 60 mg/kg/day of DE-71.

3.1.4. 2-Chloronitrobenzene

Four rats treated with 100 mg/kg/day of 2-chloronitrobenzene were observed with urine-stained abdominal fur. Slight to moderate excess salivation was observed in two rats, and clear perinasal substance was observed in one rat in this same dosage group. One additional rat in the 100 mg/kg/day dosage group had a bent tail; this observation was considered a common clinical sign in laboratory rats and was unrelated to treatment. There were no other clinical observations in rats treated with 25 or 100 mg/kg/day of 2-chloronitrobenzene.

3.1.5. Dibutyl Phthalate

Slight to extreme excess salvation was observed in three rats treated with 500 mg/kg/day and in five rats treated with 1000 mg/kg/day of dibutyl phthalate. Clear perinasal substance was observed in two rats treated with 1000 mg/kg/day of dibutyl phthalate, and one rat in each of the 500 and 1000 mg/kg/day dosage groups were observed with a bent tail, a common clinical sign in laboratory rats. There were no other clinical observations in rats treated with 500 or 1000 mg/kg/day of 2-chloronitrobenzene.

3.2. Necropsy Observations (Individual Data - APPENDIX 2)

3.2.1. Corn Oil

One rat in the control group had a large (2.2577 g) right testis. No additional gross lesions were revealed by necropsy examination of rats in this dosage group.

3.2.2. Vinclozolin

No gross lesions were revealed by necropsy examination of the rats treated with 30 or 100 mg/kg/day of vinclozolin.

3.2.3. DE-71

No gross lesions were revealed by necropsy examination of the rats treated with 30 or 60 mg/kg/day of DE-71.

3.2.4. 2-Chloronitrobenzene

A dark red spleen was observed in one and two rats treated with 25 and 100 mg/kg/day of 2-chloronitrobenzene, respectively. A black spleen was observed in two rats and an enlarged black spleen was observed in seven rats treated with 100 mg/kg/day of 2-chloro-nitrobenzene. No additional gross lesions were revealed by necropsy examination of the rats in these dosage groups.

3.2.5. Dibutyl Phthalate

Both testes were small in 10 and 14 rats, both epididymides were small in 4 and 9 rats, and the seminal vesicles (with and without fluid) were small in 1 and 3 rats treated with 500 and 1000 mg/kg/day of dibutyl phthalate, respectively. No additional gross lesions were revealed by necropsy examination of the rats treated with 500 or 1000 mg/kg/day of dibutyl phthalate.

3.3. Body Weights (Figures 1 through 4; Summary - Table 1; Individual Data - APPENDIX 3)

3.3.1. Vinclozolin

Body weights were significantly reduced ($p \leq 0.05$) on PNDs 51, 52 and 53 by dosages of 100 mg/kg/day of vinclozolin. No other biological or statistical differences occurred in the groups treated with 30 or 100 mg/kg/day of vinclozolin.

3.3.2. DE-71

Body weights of the juvenile male rats were unaffected by dosages of 30 or 60 mg/kg/day of DE-71. No biological or statistical differences occurred in the groups treated with 30 or 60 mg/kg/day of DE-71.

3.3.3. 2-Chloronitrobenzene

Body weights were significantly reduced ($p \leq 0.05$ or $p \leq 0.01$) on PNDs 51, 52 and 53 by dosages of 100 mg/kg/day of 2-chloronitrobenzene. No other biological or statistical differences occurred in the groups treated with 25 or 100 mg/kg/day of 2-chloro-nitrobenzene.

3.3.4. Dibutyl Phthalate

Body weights were significantly reduced ($p \leq 0.05$ or $p \leq 0.01$) on PNDs 45 and 47 to 53 by dosages of 1000 mg/kg/day of dibutyl phthalate. No other biological or statistical differences occurred in the groups treated with 500 or 1000 mg/kg/day of dibutyl phthalate.

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

3.4.1. Corn Oil

Mean terminal body weights for the male rats in the corn oil dosage group were 319.2 g, with a coefficient of variation of 5.8%. The initial mean body weight of these rats was 67.0 g (coefficient of variation of 8.3 %), resulting in a body weight gain of 252.2 g (coefficient of variation of 6.9%) over the course of the study.

3.4.2. Vinclozolin

Mean terminal body weights were significantly reduced by dosages of 100 mg/kg/day of vinclozolin when analyzed by an ANOVA ($p \leq 0.05$) and when analyzed by the ANCOVA ($p \leq 0.01$) using the body weight on PND 21 as the covariate. There were no biological or statistical differences in terminal body weights for rats treated with 30 mg/kg/day of vinclozolin.

When statistically analyzed by an ANOVA, the absolute weight of the left testis was significantly increased ($p \leq 0.05$) in rats treated with 100 mg/kg/day of vinclozolin. When the left testis weight was analyzed by ANCOVA using the body weight on PND 21 as the covariate, the weight was significantly increased ($p \leq 0.05$ and $p \leq 0.01$, respectively) in the rats treated with 30 mg/kg/day and in the rats treated with 100 mg/kg/day of vinclozolin.

The weight of the androgen-dependent tissue seminal vesicles (with fluid) was significantly reduced ($p \leq 0.05$) in rats treated with 100 mg/kg/day of vinclozolin, when

analyzed by ANOVA. Seminal vesicle weights (with fluid) were also significantly reduced ($p \leq 0.01$) in this dosage group when this weight was analyzed by ANCOVA using the body weight on PND 21 as the covariate; the weight of the seminal vesicles without fluid was also significantly reduced ($p \leq 0.05$) in this dosage group when analyzed by ANCOVA. Treatment of the rats with 30 mg/kg/day of vinclozolin did not affect the weight of the seminal vesicles (with or without fluid).

The absolute weights of the epididymides (paired) were significantly reduced in rats treated with 100 mg/kg/day of vinclozolin, when analyzed by ANCOVA ($p \leq 0.05$) using the body weight on PND 21 as the covariate. Dosages of 30 mg/kg/day of vinclozolin did not affect the weight of the epidiymides (paired).

The absolute weights of the adrenals (paired) in rats treated with either 30 or 100 mg/kg/day of vinclozolin were comparable to the control group value when analyzed by ANOVA, but significantly increased ($p \leq 0.05$) in rats treated with 100 mg/kg/day when the weight was analyzed by ANCOVA using the body weight on PND 21 as the covariate.

The weight of the levator ani (with bulbocavernosus muscles) was significantly reduced ($p \leq 0.05$) in rats treated with 100 mg/kg/day of vinclozolin when analyzed by ANCOVA, using the body weight on PND 21 as the covariate. Dosages of 30 mg/kg/day of vinclozolin did not affect the weight of the levator ani (with bulbcavernosus muscles).

Absolute weights of the right testis, pituitary, liver, kidneys (paired), thyroid (fixed) and ventral and dorsolateral prostate were unaffected by dosages of vinclozolin at 30 and 100 mg/kg/day.

3.4.3. DE-71

Terminal body weights were unaffected by dosages of 30 or 60 mg/kg/day of DE-71. There were no biological or statistical differences in terminal body weights for rats treated with 30 or 60 mg/kg/day of DE-71.

The mean absolute weights of the liver (17.8420 g and 20.5681 g, respectively) were significantly increased ($p \leq 0.01$) in rats treated with 30 mg/kg/day and in rats treated with 60 mg/kg/day of DE-71 when analyzed by ANOVA and when analyzed by ANCOVA using the body weight on PND 21 as the covariate.

The absolute weight of the left testis was significantly increased in the 30 mg/kg/day dosage group but not in the 60 mg/kg/day dosage group when analyzed by ANOVA ($p \leq 0.05$) and when analyzed by ANCOVA ($p \leq 0.01$), using the body weight on PND 21 as the covariate. The significant increase in the weight of the left testis was not considered treatment related because it was neither dosage dependent nor bilateral.

The absolute weights of the seminal vesicles (with and without fluid), right testis, epididymides (paired), pituitary, kidneys (paired), adrenals (paired), thyroid (fixed), ventral prostate, dorsolateral prostate and levator ani (with bulbcavernosus muscles) were unaffected by dosages of DE-71 at 30 and 60 mg/kg/day.

3.4.4. 2-Chloronitrobenzene

Terminal body weights were significantly reduced by dosages of 100 mg/kg/day of 2-chloronitrobenzene when analyzed by ANOVA ($p \leq 0.05$) and when analyzed by ANCOVA ($p \leq 0.01$) using the body weight on PND 21 as the covariate. There were no biological or statistical differences in terminal body weights for rats treated with 25 mg/kg/day of 2-chloronitrobenzene.

The absolute weight of the right testis was comparable to the control group value when analyzed by ANOVA but significantly reduced ($p \leq 0.05$) by dosages of 100 mg/kg/day of 2-chloronitrobenzene when analyzed by ANCOVA using the body weight on PND 21 as the covariate.

The absolute weights of the liver (19.5748 g and 23.5035 g, respectively) were significantly increased ($p \leq 0.01$) in rats treated with 25 mg/kg/day and in rats treated with 100 mg/kg/day of 2-chloronitrobenzene when analyzed by ANOVA and when analyzed by ANCOVA using the body weight on PND 21 as the covariate.

The absolute weight of the paired kidneys was significantly increased ($p \leq 0.01$) in rats treated with 100 mg/kg/day of 2-chloronitrobenzene when analyzed by ANOVA and by ANCOVA and significantly increased ($p \leq 0.05$) in rats treated with 25 mg/kg/day when analyzed by ANOVA and ANCOVA using the body weight on PND 21 as the covariate.

The absolute weight of the ventral prostate was comparable to the control group value in the 25 and 100 mg/kg/day dosage groups when analyzed by ANOVA but significantly reduced ($p \leq 0.05$) by dosages of 100 mg/kg/day of 2-chloronitrobenzene when analyzed by ANCOVA using the body weight on PND 21 as the covariate.

The absolute weight of the levator ani (with bulbcavernosus muscles) were significantly reduced in rats treated with 100 mg/kg/day of 2-chloronitrobenzene when analyzed by ANOVA ($p \leq 0.05$) and when analyzed by ANCOVA ($p \leq 0.01$) using the body weight on PND 21 as the covariate. There were no biological or statistical differences in the weight of the levator ani (with bulbcavernosus muscles) for rats treated with 25 mg/kg/day of 2-chloronitrobenzene.

The absolute weights of the left testis, seminal vesicles (with and without fluid), epididymides (paired), pituitary, adrenals (paired), thyroid (fixed) and dorsolateral prostate were unaffected by dosages of 2-chloronitrobenzene at 25 and 100 mg/kg/day.

3.4.5. Dibutyl Phthalate

Terminal body weights were significantly reduced ($p \leq 0.01$) in rats treated with 1000 mg/kg/day of dibutyl phthalate when analyzed by ANOVA and significantly reduced ($p \leq 0.05$ and $p \leq 0.01$, respectively) in rats treated with 500 mg/kg/day and in rats treated with 1000 mg/kg/day of dibutyl phthalate when analyzed by ANCOVA using the body weight on PND 21 as the covariate.

The absolute weights of the testes (left and right) were significantly reduced ($p \leq 0.01$) in rats treated with 500 mg/kg/day and in rats treated with 1000 mg/kg/day of dibutyl phthalate when analyzed by ANOVA and by ANCOVA using the body weight on PND 21 as the covariate.

The absolute weights of the seminal vesicles (with fluid) were significantly reduced ($p \leq 0.01$) in rats treated with 1000 mg/kg/day of dibutyl phthalate when analyzed by ANOVA. When the weights were analyzed by ANCOVA using the body weight on PND 21 as the covariate, the weights were significantly reduced ($p \leq 0.05$ and $p \leq 0.01$, respectively) in rats treated with 500 mg/kg/day and in rats treated with 1000 mg/kg/day of dibutyl phthalate. The absolute weights of the seminal vesicles (without fluid) were significantly reduced in rats treated with 1000 mg/kg/day of dibutyl phthalate when analyzed by ANOVA ($p \leq 0.05$) and by ANCOVA ($p \leq 0.01$) using the body weight on PND 21 as the covariate.

The absolute weights of the epididymides (paired) were significantly reduced in rats treated with 500 mg/kg/day and in rats treated with 1000 mg/kg/day of dibutyl phthalate when analyzed by ANOVA ($p \leq 0.05$ and $p \leq 0.01$, respectively) and by ANCOVA ($p \leq 0.05$ and $p \leq 0.01$, respectively) using the body weight on PND 21 as the covariate.

The absolute weight of the liver was significantly increased ($p \leq 0.01$) in rats treated with 1000 mg/kg/day of dibutyl phthalate when analyzed by ANOVA and by ANCOVA using the body weight on PND 21 as the covariate. There were no biological or statistical differences in liver weights for rats treated with 500 mg/kg/day of dibutyl phthalate.

The absolute weight of the levator ani (with bulbcavernosus muscles) was significantly reduced ($p \leq 0.01$) in rats treated with 1000 mg/kg/day of dibutyl phthalate when analyzed by ANOVA, as well as ($p \leq 0.05$ and $p \leq 0.01$, respectively) in rats treated with 500 mg/kg/day and in rats treated with 1000 mg/kg/day of dibutyl phthalate when analyzed by ANCOVA using the body weight on PND 21 as the covariate.

The absolute weight of the ventral prostate was significantly reduced in rats treated with 500 mg/kg/day of dibutyl phthalate when analyzed by ANOVA ($p \leq 0.05$) and by ANCOVA ($p \leq 0.01$) using the body weight on PND 21 as the covariate. The significant reduction in this organ weight for this dosage group was not considered treatment related because it was not dosage dependent; there were no biological or statistical differences in ventral prostate weights for rats treated with 1000 mg/kg/day of dibutyl phthalate.

The absolute weights of the pituitary, kidneys (paired), adrenals (paired), thyroid (fixed) and dorsolateral prostate were unaffected by dosages of dibutyl phthalate at 500 and 1000 mg/kg/day.

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

3.5.1. Corn Oil

The average age of preputial separation for the male rats administered corn oil in the control group was 41.333 days, with a coefficient of variation of 8.5%.

The average body weight at time of preputial separation for the male rats administered corn oil in the control group was 214.7 g, with a coefficient of variation of 15.8%.

3.5.2. Vinclozolin

The average age of preputial separation was significantly increased ($p \leq 0.01$) in a dosage-dependent manner for rats treated with 30 mg/kg/day and rats treated with 100 mg/kg/day of vinclozolin (44.6 and 48.4 days, respectively) when analyzed by ANOVA and by ANCOVA using the body weight on PND 21 as the covariate. The increases in age were 3.3 and 7.1 days greater than the control group value, respectively.

The average body weight at time of preputial separation was significantly increased ($p \leq 0.01$) in a dosage-dependent manner for rats treated with 30 mg/kg/day and rats treated with 100 mg/kg/day of vinclozolin (248.2 and 261.8 g, respectively), when analyzed by ANOVA and by ANCOVA using the body weight on PND 21 as the covariate. The increases in body weights were 33.6 g and 47.2 g, respectively.

3.5.3. DE-71

When analyzed by ANCOVA using the body weight on PND 21 as the covariate, the average age of preputial separation was significantly increased ($p \leq 0.05$) in rats treated with 60 mg/kg/day of DE-71. The increase in age in the 60 mg/kg/day dosage group was 2.1 days greater than the control group value. There were no biological or statistical differences in the average age of preputial separation for rats treated with 30 mg/kg/day of DE-71.

The average body weight at time of preputial separation was unaffected by dosages of DE-71 at 30 and 60 mg/kg/day.

3.5.4. 2-Chloronitrobenzene

When analyzed by ANCOVA using the body weight on PND 21 as the covariate, the average age of preputial separation was significantly increased ($p \leq 0.05$) in rats treated with 100 mg/kg/day of 2-chloronitrobenzene. The increase in age in the 100 mg/kg/day

dosage group was 2 days greater than the control group value. There were no biological or statistical differences in the average age of preputial separation for rats treated with 25 mg/kg/day of 2-chloronitrobenzene.

The average body weight at time of preputial separation was unaffected by dosages of 2-chloronitrobenzene at 25 and 100 mg/kg/day.

3.5.5. Dibutyl Phthalate

The average age of preputial separation was significantly increased for rats treated with 500 mg/kg/day and rats treated with 1000 mg/kg/day of dibutyl phthalate (44.000 and 43.533 days, respectively) when analyzed by ANOVA ($p \leq 0.05$) and by ANCOVA ($p \leq 0.01$) using the body weight on PND 21 as the covariate. The increases in age were 2.7 and 2.2 days greater than the control group value, respectively.

The average body weight at time of preputial separation was significantly increased ($p \leq 0.05$) in rats treated with 500 mg/kg/day of dibutyl phthalate when analyzed by ANCOVA using the body weight on PND 21 as the covariate. The increase in body weight at sexual maturation was 17.3 g greater than the control group value. There were no biological or statistical differences in the average body weight at the time of preputial separation for rats treated with 1000 mg/kg/day of dibutyl phthalate.

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

3.6.1. Corn Oil

The mean serum testosterone level for the rats administered corn oil in the control group was 2.383 (ng/mL), with a coefficient of variation of 53.7%. Mean serum thyroxine (T_4) levels were 8.384 mcg/dL, with a coefficient of variation of 8.3%, and mean serum TSH levels were 9.629 ng/dL, with a coefficient of variation of 52.6%.

3.6.2. Vinclozolin

Serum T_4 levels were significantly reduced (both $p \leq 0.01$) for rats treated with 30 mg/kg/day and rats treated with 100 mg/kg/day of vinclozolin (7.229 and 5.262 mcg/dL, respectively), compared to the control group value (8.384 mcg/dL).

Serum testosterone levels and serum TSH levels were unaffected by dosages of vinclozolin at 30 and 100 mg/kg/day.

3.6.3. DE-71

Serum T_4 levels were significantly reduced (both $p \leq 0.01$) for rats treated with 30 mg/kg/day and rats treated with 60 mg/kg/day of DE-71 (4.447 and 4.078 mcg/dL, respectively), compared to the control group value (8.384 mcg/dL).

Serum testosterone levels and serum TSH levels were unaffected by dosages of DE-71 at 30 and 60 mg/kg/day.

3.6.4. 2-Chloronitrobenzene

Serum T_4 levels were significantly increased (both $p \leq 0.05$) for rats treated with 25 mg/kg/day and rats treated with 100 mg/kg/day of 2-chloronitrobenzene (9.293 and 9.169 mcg/dL, respectively), compared to the control group value (8.384 mcg/dL).

Serum testosterone levels and serum TSH levels were unaffected by dosages of 2-chloronitrobenzene at 25 and 100 mg/kg/day.

3.6.5. Dibutyl Phthalate

Serum T_4 levels were significantly reduced (both $p \leq 0.01$) for rats treated with 500 mg/kg/day and rats treated with 1000 mg/kg/day of dibutyl phthalate (6.946 and 6.818 mcg/dL, respectively), compared to the control group value (8.384 mcg/dL).

Serum testosterone levels were unaffected by dosages of dibutyl phthalate at 500 and 1000 mg/kg/day.

Serum TSH level was significantly reduced ($p \leq 0.01$) in rats treated with 500 mg/kg/day of dibutyl phthalate. This significant reduction in serum TSH was not considered treatment related because it was not dosage dependent; there were no biological or statistical differences in serum TSH levels for rats treated with 1000 mg/kg/day of dibutyl phthalate.

3.7. Histopathology (APPENDIX 11)

Thyroids were examined microscopically and the slides were coded, making the pathologist blind to the chemical and dose group. Subjectively evaluated for follicular epithelial height and colloid area used a five point grading scale (1 = shortest/smallest; to 5 = tallest/largest). A detailed histopathological examination was conduced on the right testis and right epididymis (caput, corpus and cauda). Throughout all groups, clear cells in the caudal epididymis occurred in small incidences; their presence, or absence, was not considered significant.

3.7.1. Corn Oil

The rats assigned to the control group were the least affected concerning follicular epithelial cell height and the amount of colloid within follicles. One rat (313) had a grossly large right testis and microscopically exhibited marked cellular degeneration with enlarged lumens of the seminiferous tubules, presumably filled with fluid.

3.7.2. Vinclozolin

Follicular epithelial cell height and the amount of colloid within follicles was unaffected by treatment of rats with 30 mg/kg/day and 100 mg/kg/day of vinclozolin.

Minimal lymphocytic infiltration and/or sloughing of the epithelium in the lumen of the right epididymis was observed in rats treated with 30 mg/kg/day and rats treated with 100 mg/kg/day of vinclozolin. None of the microscopic changes were considered significant in incidence or severity.

3.7.3. DE-71

The changes seen in epithelial height and colloid area in rats treated with 30 and 60 mg/kg/day of DE-71 rarely exceeded a ranking score of 3. One and two rats in the 30 and 60 mg/kg/day dosage groups, respectively, had a follicular epithelium score of four. The changes seen in epithelial height and colloid area could easily be attributed to physiological variation.

Minimal lymphocytic infiltration and/or sloughing of the epithelium in the lumen of the right epididymis was observed in rats treated with 30 mg/kg/day and rats treated with 60 mg/kg/day of DE-71. None of the microscopic changes were considered significant in incidence or severity.

3.7.4. 2-Chloronitrobenzene

The changes seen in follicular epithelial cell height (minor increases) and the amount of colloid area (marginal depletion) in rats treated with 25 mg/kg/day or 100 mg/kg/day of 2-chloronitrobenzene never exceeded a ranking score of 3 and could easily be attributed to physiological variation.

Mild degeneration of the germinal epithelium of the right testis occurred in one rat treated with 25 mg/kg/day of 2-chloronitrobenzene. Minimal to mild lymphocytic infiltration and minimal sloughing of the epithelium in the lumen of the right epididymis was observed in rats treated with 25 mg/kg/day of 2-chloronitrobenzene.

Significant testicular changes, mild to moderate degeneration of the germinal epithelium of the right testis and the presence of intralumenal multinucleated giant cells, occurred in rats treated with 100 mg/kg/day of chloronitrobenzene. Mild to marked spermatic depletion and minimal to moderate sloughing of the epithelium in the lumen of the right epididymis was observed in rats treated with 100 mg/kg/day of 2-chloronitrobenzene.

3.7.5. Dibutyl Phthalate

The changes seen in follicular epithelial cell height (minor increases) and the amount of colloid area (marginal depletion) in rats treated with either 500 mg/kg/day or

1000 mg/kg/day of dibutyl phthalate never exceeded a ranking score of 3 and could easily be attributed to physiological variation.

Significant testicular changes, minimal to marked degeneration of the germinal epithelium of the right testis and/or the presence of intralumenal multinucleated giant cells, occurred in rats treated with 500 mg/kg/day of dibutyl phthalate. Minimal to marked spermatic depletion, minimal to mild lymphocytic infiltration and minimal to moderate sloughing of the epithelium in the lumen of the right epididymis was observed in rats treated with 500 mg/kg/day of dibutyl phthalate.

Significant testicular changes, mild to marked degeneration of the germinal epithelium of the right testis and the presence of intralumenal multinucleated giant cells, occurred in rats treated with 1000 mg/kg/day of dibutyl phthalate. Moderate to marked spermatic depletion and minimal to moderate sloughing of the epithelium in the lumen of the right epididymis was observed in rats treated with 1000 mg/kg/day of dibutyl phthalate.

Histopathological evaluation revealed instances at both dosages of dibutyl phthalate where seminiferous tubules were lined solely with Sertoli cells.

4. DISCUSSION

This study was done as a step toward validating the Pubertal Male Assay as an alternative assay in the Tier I battery. The transferability of the protocol was evaluated using vinclozolin, DE-71, 2-chloronitrobenzene and dibutyl phthalate, three of the chemical compounds were 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, reproductive weights and thyroid weights and histology (including colloid and follicular cell height), non-reproductive organ weights (liver, kidney, pituitary, and adrenals) and systemic hormone concentrations (testosterone, T_4 and TSH), in part, in response to vinclozolin, DE-71, 2-chloronitrobenzene or dibutyl phthalate exposure.

Three of the chemicals tested caused general toxicity limited to a few adverse clinical observations, mainly at the highest dosage tested for each chemical and reduced body weights at the end of the treatment period. There were no deaths on the study. Urine-stained abdominal fur, excess salvation and clear perinasal substance was observed in rats treated with 100 mg/kg/day of 2-chloronitrobenzene. Excess salivation substance was observed in rats treated with 500 and 1000 mg/kg/day and clear perinasal was observed in rats treated with 1000 mg/kg/day of dibutyl phthalate. There were no other clinical observations that were considered treatment related in the groups treated with vinclozolin or DE-71.

Two chemicals caused observable necropsy findings. A dark red or black spleen and enlarged spleen was observed in rats treated with 25 or 100 mg/kg/day of 2-chloro-nitrobenzene. Testes, epididymides and seminal vesicles were small in rats treated with 500 or 1000 mg/kg/day of dibutyl phthalate. There were no other necropsy observations that were considered treatment related in the dosage groups treated with vinclozolin or DE-71.

Mean body weights were significantly reduced by 100 mg/kg/day of vinclozolin, 100 mg/kg/day of 2-chloronitrobenzene, and 1000 mg/kg/day of dibutyl phthalate exposures. There were no significant differences in the mean body weight between the control group, which was administered corn oil, and the groups treated with DE-71.

The four chemicals at the doses tested had effects on the reproductive organ weights (testes, seminal vesicles, levator ani and prostate) and liver, kidneys and/or spleen weights. The absolute weights of the left testis were significantly increased in rats treated with 30 and 100 mg/kg/day of vinclozolin. The absolute weights of the adrenals were significantly increased and the absolute weight of the seminal vesicles and levator ani were reduced in rats treated with 100 mg/kg/day of vinclozolin. The absolute weights of the epididymides (paired) were significantly reduced in rats treated with 100 mg/kg/day of vinclozolin. The mean absolute weights of the liver were significantly increased by the 30 and 60 mg/kg/day dosages of DE-71. The absolute weight of the right testis was significantly reduced in rats treated with 100 mg/kg/day of choronitrobenzene. The absolute weights of the liver were significantly increased in rats treated with 25 and 100 mg/kg/day of 2-chloronitrobenzene. The absolute weight of the paired kidneys was significantly increased in rats treated with 25 and 100 mg/kg/day of 2-chloro-nitrobenzene. The absolute weights of the ventral prostate were significantly reduced in rats treated with 100 mg/kg/day of 2-chloronitrobenzene and the absolute weight of the levator ani were significantly reduced in rats treated with 100 mg/kg/day of 2-chloro-nitrobenzene. The absolute weights of the testes, seminal vesicles, epididymides and levator ani were significantly reduced, in rats treated with 500 and 1000 mg/kg/day of dibutyl phthalate. The absolute weight of the liver was significantly increased in rats treated with 1000 mg/kg/day of dibutyl phthalate. There were no differences in thyroid weights for any of the test substances when compared to control value analyzed using either ANOVA or ANCOVA, with the body weight on PND 21 as the covariate.

The chemicals tested did affect sexual maturation. Both the average age of preputial separation and average body weight at time of preputial separation were significantly increased in a dosage-dependent manner in rats treated with 30 and 100 mg/kg/day of vinclozolin and in rats treated with 500 mg/kg/day of dibutyl phthalate. The average age of preputial separation was significantly increased in rats treated with 60 mg/kg/day of DE-71, 100 mg/kg/day of 2-chloronitrobenzene and 1000 mg/kg/day of dibutyl phthalate.

All four chemicals caused changes in serum T_4 levels, and none affected testosterone or TSH levels. Serum T_4 levels were significantly reduced in rats treated with 30 and 100 mg/kg/day of vinclozolin, 30 and 60 mg/kg/day of DE-71 and 500 and

1000 mg/kg/day of dibutyl phthalate. Serum T_4 levels were significantly increased in rats treated with 25 and 100 mg/kg/day of 2-chloronitrobenzene. Serum testosterone levels and serum TSH levels were unaffected by dosages of 30 and 100 mg/kg/day of vinclozolin, 30 and 60 mg/kg/day of DE-71, 25 and 100 mg/kg/day of 2-chloro-nitrobenzene, and 500 and 1000 mg/kg/day of dibutyl phthalate.

None of the four chemicals tested had a significant effect on the histopathology of the thyroid, and two chemicals had a histological effect on the reproductive system. The rats treated with 30 and 100 mg/kg/day of vinclozolin showed no effects on the thyroid; there were no significant signs of testicular or epididymal atrophy in any of the rats evaluated. The changes seen in epithelial height and colloid area in rats treated with 30 and 60 mg/kg/day of DE-71 rarely exceeded a ranking score of 3. One and two rats in the 30 and 60 mg/kg/day dosage groups, respectively, had a follicular epithelium score of four. The changes seen in epithelial height and colloid area could easily be attributed to physiological variation; there were no significant signs of testicular or epididymal atrophy in any of the rats evaluated. Significant testicular changes, degeneration of the germinal epithelium and the presence of intralumenal multinucleated giant cells, occurred in the rats treated with 100 mg/kg/day of 2-chloronitrobenzene, as well as spermatic depletion and sloughing of the epithelium in the lumen of the right epididymis. The rats treated with 500 and 1000 mg/kg/day of dibutyl phthalate showed minor thyroid effects and minor increases in follicular epithelial cell height and marginal colloid depletion in the follicles. The changes seen in epithelial height and colloid area never exceeded a ranking score of 3 and could easily be attributed to physiological variation. Significant testicular changes where seminiferous tubules were lined only with Sertoli cells occurred in rats treated with 500 and 1000 mg/kg/day of dibutyl phthalate; significant testicular changes, degeneration of the germinal epithelium and the presence of intralumenal multinucleated giant cells, occurred in the rats treated with 500 and 1000 mg/kg/day of dibutyl phthalate, as well as spermatic depletion and sloughing of the epithelium in the lumen of the right epididymis.

5. CONCLUSION

The four test chemicals and their target/mechanism of action were as follows:
1) vinclozolin - an anti-androgen through competitive binding of metabolites M1 and M2 to the androgen receptor, M1 binds weakly to the rat progesterone receptor; 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;
3) 2-chloro-nitrobenzene - a nitro-aromatic, inducing methemoglobinemia; and 4) dibutyl phthalate - an anti-androgen through the androgen or β-estrogen receptor[15].

Vinclozolin is a protectant, non-systemic dicarboximide fungicide. The USEPA considers vinclozolin to be an endocrine-disrupting chemical, interfering with lipid metabolism and/or storage and inducing reduced sperm count, decreased prostate weight and delayed puberty in test animals[16]. The UK Advisory Committee on Pesticides specific concern is that vinclozolin could feminize and damage the reproductive capacity

of male rats[17]. In this study, 30 and 100 mg/kg/day of vinclozolin administered to juvenile male rats for 31 consecutive days did demonstrate anti-androgen activity: it caused reduced body weights (100 mg/kg/day only), changes in the weights of the reproductive organs (100 mg/kg/day only), increased (delayed) the age of sexual maturation, decreased serum T_4 levels, but had no effect on necropsy or histopathological findings.

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 at similar dose levels to cause decreased serum T_4 levels, thyroid changes, increased liver weight, reduced seminal vesicle and ventral prostate weight, and delays in preputial separation[18]. In the current study, administration of DE-71 at 30 and 60 mg/kg/day to juvenile male rats for 31 consecutive days did demonstrate thyroid activity: it caused significant increases in liver weights, increased (delayed) the age of sexual maturation (60 mg/kg/day only), reduced serum T_4 levels, minor increases in follicular epithelial cell height and marginal colloid depletion in the thyroids, but showed no effect on reproductive organ weights or necropsy findings and caused no significant histopathological findings.

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 increases in liver and spleen weights with no reproductive toxicity[19, 20]. In the current study, administration of 2-chloronitrobenzene at 25 and 100 mg/kg/day to juvenile male rats for 31 consecutive days did demonstrate thyroid activity: it caused minor increases in follicular epithelial cell height and marginal colloid depletion in the thyroids and increased serum T_4 levels. Other systemic toxicity included adverse clinical signs, enlarged and discolored spleens, reduced body weights (100 mg/kg/day only), increased kidney and liver weights, changes in reproductive organ weights [reductions in testis, ventral prostate and levator ani weights (100 mg/kg/day only)], increased (delayed) the age of sexual maturation (100 mg/kg/day only), increases in serum T_4 levels and in histopathological findings (100 mg/kg/day only).

Dibutyl phthalate is used in making flexible plastics that are found in a variety of consumer products such as shower curtains, raincoats, food wraps, bowls, car interiors, vinyl fabrics, floor tiles, and other products[21]. Animal studies have reported developmental effects, such as reduced fetal weight, decreased number of viable litters, and birth defects (neural tube defects) in mice exposed orally to dibutyl phthalate. Reproductive effects, such as decreased spermatogenesis and testes weight, have also been reported in oral animal studies [20, 21]. In this study, 500 and 1000 mg/kg/day dibutyl phthalate administered to juvenile male rats for 31 consecutive days, did demonstrate anti-androgen activity: it caused changes in the weights of the reproductive organs (reduced testes, seminal vesicles, epididymides and levator ani weights), increased (delayed) the age of sexual maturation, and significant testicular changes, including instances where seminiferous tubules were lined only with Sertoli cells. Other systemic

toxicity included adverse clinical signs and necropsy observations, reduced body weights (100 mg/kg/day only), increased liver weights (100 mg/kg/day only), minor increases in follicular epithelial cell height and marginal colloid depletion in the thyroids and reduced serum T_4 levels.

3-JAN-06

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

23 JAN 06

Raymond G. York, Ph.D., DABT Date
Associate Director of Research
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7. PROTOCOL DEVIATIONS

1. The incorrect lot number of the Dibutyl Phthalate was used. The protocol stated that lot number 00323P4 was to be used and lot number 00323PU was used. 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.

2. The storage conditions 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.

3. 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.

4. 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.

5. The Testing Facility reserve sample of vinclozolin was 0.56 g, rather than 1.0 g as specified in the protocol. This deviation did not adversely affect the outcome or interpretation of the study because this did not result in the loss of any data.

6. 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.

7. Neither partial preputial separation nor the presence of a persistent thread between the glans and the prepuce was recorded for the duration of the study. This deviation did not adversely affect the outcome or interpretation of the overall study because: 1) all male rats on study passed sexual maturation prior to sacrifice and 2) the study encompassed more than just sexual maturation endpoints.

8. The following rats did not have gross lesions saved in neutral buffered 10% formalin (NBF) on the day of sacrifice.

Animal Number	Dosage Group	Gross Lesion
406	G	Left testis
407	G	Left testis
410	G	Left testis
418	G	Left testis
420	G	Left testis
421	H	Left testis
422	H	Left testis
429	H	Seminal vesicles
432	H	Left epididymis and testis

These deviations did not adversely affect the outcome or interpretation of the study because sufficient data were collected in order to evaluate this parameter.

9. All gross lesions were not histologically evaluated. Only gross lesions associated with the right testis, epididymis 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.

10. The blood samples collected on 30 April 2005 and 01 May 2005 were stored at -70ºC in order to maintain sample integrity. The manufacturer indicated that the samples could be stored at -20ºC for several days. This deviation did not adversely affect the outcome or interpretation of the study because there is no indication that storage of the samples below -20ºC could be detrimental and all samples were treated the same.

11. There was no documentation of the storage conditions of blood samples analyzed on 17 May 2005 and no documentation of bringing the blood samples to room temperature prior to analysis. This deviation did not adversely affect the outcome or interpretation of the study because the results of the analysis in relation to other samples indicate that the samples were stored and handled properly.

12. 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 by the Sponsor on 29 August 2005.

All deviations are documented in the raw data.

03 JAN 06

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