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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

013951

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

12/20/99

SUBJECT: Atrazine: Review of a method validation study which evaluated a method of measuring the proestrus afternoon LH and prolactin surges in Sprague-Dawley female rats.

FROM: Roger Hawks, Ph.D. Roger Hawks 12/20/99
Reregistration Branch III
Health Effects Division (7509C)

THRU: Jess Rowland, Branch Chief Jess Rowland 1/16/00
Reregistration Branch III
Health Effects Division (7509C)

TO: Pam Noyes
Special Review and Reregistration Division (7508C)

DP Barcode: D261336
Submission No.: S571786
Chemical: 080803
Caswell No.: 063

Purpose of Memo - A method validation study (MRID 43934405) was performed in order to determine the validity of a protocol to be used in subsequent studies to measure the proestrus afternoon LH and prolactin surges in the Sprague-Dawley female rat. This study was reviewed and a data evaluation record (DER) of this study was written. The study was found to be **Acceptable-nonguideline**. The study does not satisify a FIFRA Subdivision F or OPPTS series 870 guideline and was not submtted withthe intention of doing so.
This study adequately demonstrates the validity of the methods proposed for evaluating LH and prolactin surges.

Primary Reviewer- Roger Hawks, Ph.D., RRBIII
Secondary Reviewer - Melba Morrow, D.V.M., RABI

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The DER is attached and the executive summary follows:

EXECUTIVE SUMMARY:

In a study (MRID 43934405) to determine the validity of a proposed protocol for testing the effect of atrazine exposure on the proestrus afternoon luteinizing hormone (LH) surge, atrazine was administered, by gavage, to 20 ovariectomized (OVX) female Sprague Dawley rats which had a surgically implanted estradiol (E_2)-containing silastic capsule. Serum LH and prolactin levels were measured following 3 days of exposure to 300 mg/kg/day of atrazine to determine if the LH and prolactin surges could be measured in atrazine-exposed animals.
Control animals displayed the expected LH and prolactin surges. This confirms the results of MRID 43934404 that these hormone surges can be induced by implantation of an estradiol-containing pellet in OVX SD females. The prolactin surge was not altered in the atrazine-exposed animals. The LH surge was not attenuated in the atrazine exposed animals but was delayed by approximately two hours.

This special study in the rat is **Acceptable-nonguideline**. This study does not satisfy any guideline requirements and was not submitted with the intention of satisfying a guideline requirement.

013951

[ATRAZINE]

Special Study - Non-Guideline

EPA Reviewer: Roger Hawks
Reregistration Branch III(7509C)
EPA Secondary Reviewer: Melba Morrow, D.V.M.
(7509C)

Roger Hawks, Date 12/15/99

Melba Morrow, Date 12/17/99

DATA EVALUATION RECORD

DP BARCODE: D261336
P.C. CODE: 080803

SUBMISSION CODE: S571786
TOX. CHEM. NO.: 063

TEST MATERIAL: Atrazine (97.1%)

CITATION: Morseth, S. (1996) Evaluation of luteinizing hormone (LH) in female Sprague-Dawley rats- method validation. Corning Hazelton Inc., Vienna, VA. Laboratory report number: CHV 2386-110. January 18, 1996. MRID:43934405. Unpublished.

SPONSOR: Novartis Corporation (formerly Ciba-Crop Protection) Greensboro, N.C.

EXECUTIVE SUMMARY:

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COMPLIANCE: Signed and dated GLP, Quality Assurance, Data Confidentiality, and Flagging statements were provided.

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I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material : atrazine
   Description: white powder
   Lot#: SG8029BAI0
   Purity: 97.1% a.i.
   Stability of compound: Stable at room temperature

2. Test Material Vehicle and Positive control: 0.05% Carboxymethylcellulose (CMC). Lot no. 23H0244

3. Estradiol : β-Estradiol 3-benzoate
   Description: white powder
   Lot#: 52H3881
   Purity: 98% a.i.
   Stability of compound: Stable at room temperature

4. Estradiol Vehicle: Sesame Oil
   Description: clear, yellowish liquid
   Lot#: 1113H1212
   Stability of compound: Stable at room temperature

5. Test animals: Species: Rat
   Strain: Sprague-Dawley
   Age and weight at study initiation: 8 weeks; Weight at initiation not given
   Source: Charles River Labs, Raleigh, N.C.
   Housing: During the study the animals were individually housed in stainless steel wire-mesh cages.
   Diet: PMI® Feeds, Inc. Certified Rodent Diet #5002. ad libitum
   Water: Tap water ad libitum
   Environmental conditions:

   Temperature: 64.4 to 78.3 °F.
   Humidity: 41.3 to 85.4%
   Air changes: ≥ 10 per hour
   Photoperiod: 14 hour light/10 hour dark

   Acclimation period: Five weeks

B. STUDY DESIGN:

1. In life dates - start: July 17, 1995 end: July 24, 1995

2. Animal assignment - Animals were assigned using randomly generated numbers from a computer to dose groups shown in Table 1.

TABLE 1: Test Groups

Test Group	Dose Level (mg/kg/day)	Number of females
1	0	10
2	300	10

Table 2: Study Timetable

Study Initiation	July 17, 1995
Ovareiectomy	July 21, 1995
Atrazine Administration	July 22, 23 and 24, 1995
Sacrifice	July 24, 1995

3. Rationale and purpose of study - This study was designed to validate the procedures used to measure proestrus afternoon LH and prolactin surges. This study is one of a series of studies (MRIDs 43934404; 43934405; 43934406; and 44152102) which examine the effect of atrazine exposure on, primarily, the proestrus afternoon LH surge. This hormonal surge stimulates ovulation. A delay or lack of ovulation in response to atrazine exposure has been implied in a previous study (MRID 42085001) which examined estrous cycles (through vaginal smears) in SD rats exposed to atrazine. SD rats in this study displayed an increased percentage of days in the estrus phase of the estrous cycle early in the study following atrazine exposure. Increased days in estrus implies a delay or lack of ovulation. A histomorphic evaluation of the ovaries from this study (MRID 43598622) also showed evidence of a delay or absence of ovulation at the early time points in atrazine-exposed SD females.

The proestrus afternoon LH surge was investigated in this series of studies to examine the possibility that the apparent delay or absence of ovulation seen in MRID 42085001 was due to atrazine's effects on the LH surge.

In addition to the proestrus afternoon LH surge, rats also display a proestrus afternoon prolactin surge. The effects of atrazine exposure on this surge were also investigated in this series of studies.

The effects of atrazine on the LH and prolactin surges are investigated in MRIDs

43934406 and 44152102. The current study (MRID 43934404) and MRID 43934405 - which is reviewed in a separate DER - are, respectively, a pilot study and method validation study in which the methods and protocol used to measure the LH and prolactin surges are tested and validated.

The specific procedures validated in this study were:

- The effectiveness of implantation of silastic estradiol pellets in raising serum estradiol levels to a level sufficient to stimulate an LH surge;
- The effect of repeated bleeding on hormone levels;
- The utility of the proposed method of blood collection and hormone measurement (jugular vein bleed, plasma fractionation, freezing at -70°, measurement by radioimunoassay).

The animals were ovariectomized (OVX) in this study. The purpose of OVX followed by implantation of an estradiol-containing pellet is described in the DER for MRID 43934404.

5. Formulation of Test Article

The 0.5% CMC vehicle was prepared by adding an appropriate amount of CMC into distilled water and stirring until the CMC was fully dissolved. A 4 mg/ml atrazine formulation was then prepared by adding atrazine to a small amount to the 0.5% CMC. This paste was bought to 3/4ths volume and stirred in a beaker for five minutes. This was then bought to full volume and stirred for another five minutes. the formulation was then aliquoted and stored under refrigeration.

6. Statistics - A repeated measures Analysis of Variance (ANOVA) was used to analyze the LH data. The prolactin data was not analyzed statistically.

C. METHODS:

1. Observations:

Animals were inspected once a day prior to ovariectomy and twice daily for signs after OVX for signs of toxicity and mortality.

2. Body weight

Body weights were to be taken once prior to surgery.

3. Food consumption

Neither the study report nor the study protocol note that food consumption was to be determined.

4. Estradiol capsule preparation

Estradiol was added to sesame oil to a concentration of 4 mg/mL and stirred in a beaker heated to 80°C until a solution was formed. This solution was added to a 20 mm in length silastic tubing with an active length of 8 mm. The ends of the tubing were sealed to make the estradiol-containing capsule. The final concentration of estradiol in the capsules was 4 mg/mL.

5. Ovariectomization

The animals were anesthetized under isoflurane inhalation anesthetic and the ovaries were surgically removed through lateral-dorsal incisions. Surgical wounds were closed with wound clips and Vicryl sutures as needed.

6. Estradiol capsule implantation

The estradiol capsules were surgically implanted at the time of ovariectomy.

7. Atrazine Administration

The atrazine formulation was removed from the refrigerator approximately one hour before it was to administered and was stirred. The atrazine was delivered by oral gavage at a dose volume of 10 mL per kg of body weight. Atrazine exposure began on the day immediatly OVX. Animals were gavaged for three consecutive days and were sacrificed following exposure on the third day.

8. Sacrifice and Blood Collection

Blood collection occurred on the third day of atrazine exposure. Blood (0.8 to 1 mL) was collected from the jugular vein into sodium heparin-containing tubes. Blood collection occurred following the schedule shown in Table 3. The tubes were refrigerated at 4°C until they could be spun down for collection of the plasma fraction. The difference between clock time and biologic time is explained in the DER for MRID 43934404. It is sufficient to know that the animals were bled over a course of time in which it would be expected that the LH and prolactin surges would be rising, at a peak and then returning to baseline.

Table 3: Schedule for Blood Collection

Clock hour	Biologic Timepoint
7:00 am	1100
9:00 am	1300
11:00 am	1500
2:00 pm	1800
4:00 pm	2200

9. Hormone measurements

Plasma was analyzed by radioimunoassay for LH and prolactin at Covance.

10. Necropsy and Histopathology

Neither macroscopic nor histologic examinations were performed.

II. **RESULTS**:

1. Body weights

Body weights were not reported.

2. LH levels

An LH surge was evident in both the control and treated animals. The surge in the atrazine-treated animals may have been delayed. At 1500 hours in the controls the surge has clearly begun, but at 1500 hours in the treated animals LH values are within 10% of baseline values. Both groups show clear surges at 1800 and 2200 hours; indicating that the surge in the treated groups was only delayed, not absent. LH data are displayed in Tables 4 and 5 below and in Figure One. Though a surge seems evident in both groups of animals, there were no statistically significant differences between controls *vs* treated or between timepoints within each group.

Table 4: Plasma LH levels by time point in controls. Values given in ng/mL.

1100	1300	1500	1800	2200
$\bar{x}$= 0.53 SD= 0.198	$\bar{x}$= 0.599 SD= 0.493 %[1]= +12%	$\bar{x}$= 0.977 SD= 1.04 %= +84%	$\bar{x}$= 1.309 SD= 1.23 %= +146%	$\bar{x}$= 1.225 SD= 0.85 %= +131%

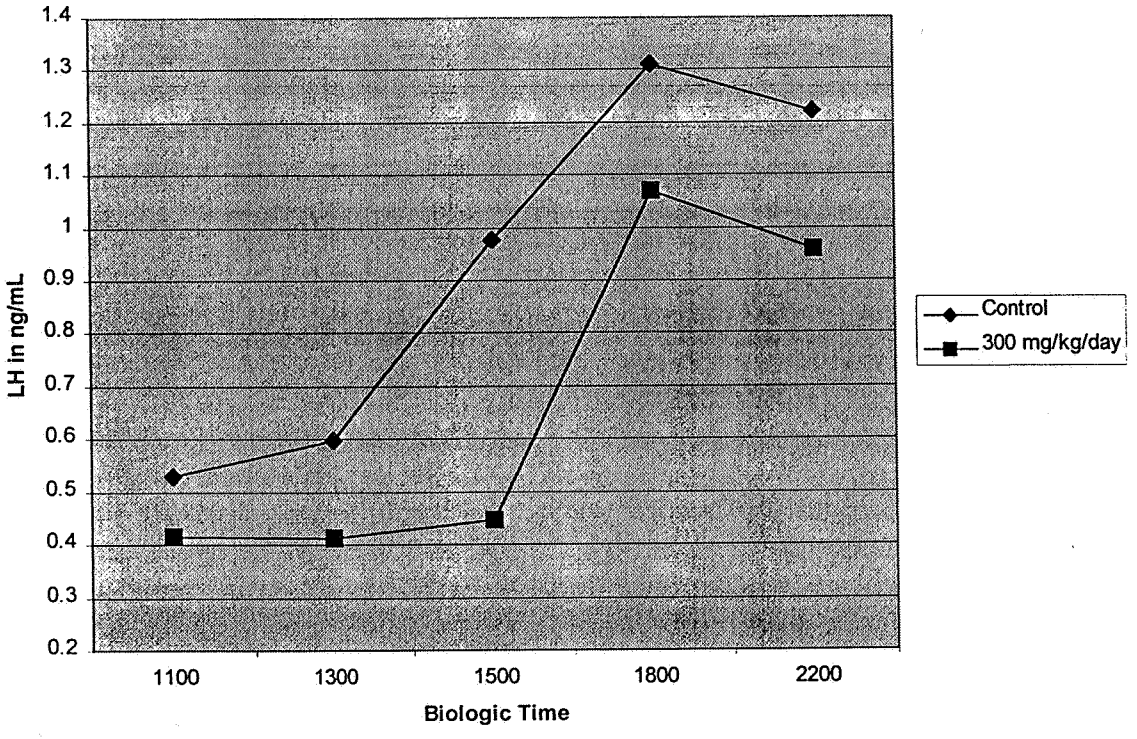
1 Represents the percentage change from baseline (1100 hours)
Data from page 22, current study

Table 5: Plasma LH levels by time point in animals treated with 300 mg/kg/day atrazine. Values given in ng/mL.

1100	1300	1500	1800	2200
$\bar{x}$= 0.414 SD= 0.08	$\bar{x}$= 0.413 SD= 0.108 %[1]= 0%	$\bar{x}$= 0.446 SD= 0.178 %= +7.7%	$\bar{x}$= 1.07 SD= 1.11 %= +158%	$\bar{x}$= 0.961 SD= 0.83 %= +132%

1 Represents the percentage change from baseline (1100 hours)
Data from page 23, current study

Figure One: LH Measurements



3. Prolactin levels

A prolactin surge was evident in both control and treated animals. Prolactin data are displayed in Tables 6 and 7 below and in Figure Two.

Table 6: Plasma prolactin levels by time point in controls. Values given in ng/mL.

1100	1300	1500	1800	2200
$\bar{x}$= 62.89 SD= 104.76	$\bar{x}$= 77.98 SD= 99.71 %[1]= +24%	$\bar{x}$= 171.5 SD= 227.55 %= +173%	$\bar{x}$= 276.11 SD= 192.65 %= +340%	$\bar{x}$= 202.15 SD= 130.38 %= +221%

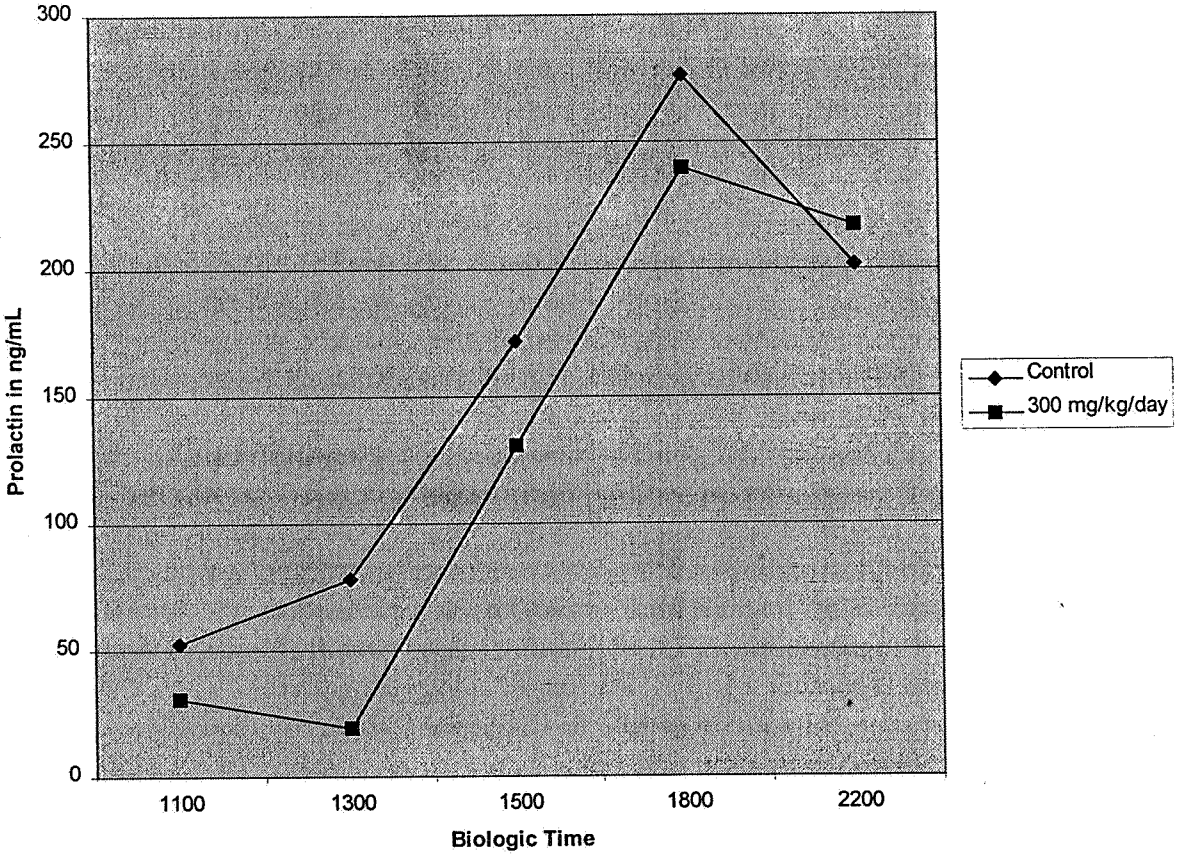
1 Represents the percentage change from baseline (1100 hours)
Data from page 24, current study

Table 7: Plasma prolactin levels by time point in animals treated with 300 mg/kg/day atrazine. Values given in ng/mL.

1100	1300	1500	1800	2200
$\bar{x}$= 30.86 SD= 34.04	$\bar{x}$= 19.48 SD= 9.5 %[1]= -37%	$\bar{x}$= 131.01 SD= 203.9 %= +287%	$\bar{x}$= 239.51 SD= 209.91 %= 676%	$\bar{x}$= 217.57 SD= 171.9 %= +605%

1 Represents the percentage change from baseline (1100 hours)
Data from page 25, current study

Figure Two: Prolactin Measurements



III. DISCUSSION

A. This study confirmed the findings from MRID 43934404 that OVX followed by implantation of an estradiol-containing pellet will induce an LH surge in SD females. This study determined that repeated bleeding (bleeding the same animals over and over again at each timepoint) is a valid method to use to measure LH and prolactin surges. The study author commented that the repeated bleed protocol was appropriate for examination of the LH surge, but not the prolactin surge. The study author states that plasma prolactin levels did not drop back to baseline levels because the animals had been stressed by the repeated bleeding and stress is known to raise blood prolactin levels. The reviewer acknowledges that stress can raise prolactin levels and that plasma prolactin levels did not drop in this study. The reviewer does not believe there is sufficient evidence to conclude that the lack of a return to baseline for the prolactin levels indicates stress upon the animal. Although failing to return to baseline levels, mean plasma prolactin levels did drop in the last timepoint. LH levels also failed to return to baseline. The reason neither plasma LH nor prolactin failed to return to baseline levels may be simply because there was not enough time for them to do so before the final timepoint. Peak LH and prolactin values were seen at 1800 hours in this study. The final hormone measurement was at 2200 hours - only 4 hours following the peak. In MRID 43934404, peak hormone levels were seen at 1600 hours for LH and at 1400 for prolactin. In this study hormone levels did not return to baseline (≤20% of baseline values) until 2000 hours- 6 hours following the prolactin peak and 4 hours following the LH peak. Prolactin levels in this study may very well have been found to return to baseline had there been additional timepoints beyond 2200 hours.
The lack of a return of prolactin levels to baseline is also not of concern, in this study, because a large variability in this type of data would be expected. Measuring levels of hormones that are rapidly raising and falling, as is done in the case of the LH and prolactin surges, is bound to result in inherent data variability.
This study is classified as Acceptable-Non-Guideline.

This study confirmed the effectiveness of implantation of silastic estradiol pellets in raising serum estradiol levels to a level sufficient to stimulate an LH surge; failed to find any firm evidence that repeated bleeding alters hormone levels; and, demonstrates the validity of blood collection and hormone measurement by jugular vein bleed, plasma fractionation, freezing at -70°, and radioimunoassay.

B. Study deficiencies

This study contained minor deficiencies. Body weights do not appear to have been used to randomize the animals. Body weights were not reported. A lack of hormone measurements at timepoints beyond 4 hours after peak hormone levels were seen complicates interpretation of the hormone data. These deficiencies do not alter the classification of this study as Acceptable-Non-Guideline.