9-15-94

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

SEP 15 1994

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OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM:

Subject: EPA ID# 113201: Vinclozolin, DER on the Hormone Status of Rats After 90-Days Dosing and Untreated Recovery (MRID# 427061-02) and Some Androgen Binding Studies with Human Cell Lines and Prostate Tissue (MRID# 527061-01).

Barcode: 192566. ToxChem No.: 323C.
Submission No.: S443224. PC No.: 113201
MRID No.: 427061-01 & -02. Case No.: 011409.

From: David G Anderson, PhD David G Anderson 9/1/94.
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Thru: Karen Hamernik, PhD K.H. 9/1/94 KH 9/9/94
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The following studies were submitted in support of the reregistration of vinclozolin by BASF.

MRID# 427061-02:

1) Mellert, D (December 9, 1994) Report-Study on the Influence of Reg. No. 83 258 (Vinclozolin) on the Hormonal Status of Wistar Rats; Administration in the Diet for 3 Months and a Recovery period of at least 8 Weeks in the satellite Animals. Study no. 92/11548. Conducted by BASF for BASF. MRID# 427061-02

EXECUTIVE SUMMARY: In a special study (BASF, 1992, MRID# 427061-01), vinclozolin was administered in the feed to 10 Wistar rats per sex per group to two control groups, 1 for 3 months and the other for 3 months plus 3 weeks at 0 mg/kg/day and to two dose groups, 1 for 3 months at 4500 ppm (about 300 mg/kg/day) and the other for 3 months at 4500 ppm, followed by 8 weeks of untreated

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recovery.

Several parameters were determined in these groups. Plasma levels of ACTH, LH, FSH, testosterone, corticosterone, aldosterone, DHEA and estradiol were determined in the two control groups and in the two dose groups. Cytochrome P450 was determined in the livers of the same respective groups of animals. Urine volumes, urinary ketosteroids and adrenal and liver organ weights were also determined in the same respective groups of animals. Estrous cycles were determined in the females of the 4 respective groups for the 3 to 4 weeks before termination.

Plasma was obtained from males and females by decapitation; females were in diestrus at the time of decapitation. ACTH (178% of controls), LH (940% of controls), FSH (215% of controls), testosterone (276% of controls), DHEA (189% of controls) and may be corticosterone (160% of controls) and aldosterone (154% of controls) in males were elevated after 3 months of dosing at 4500 ppm, but returned to control values during the 8 week recovery. ACTH (255% of controls) and LH (250% of controls) were elevated in females after 3 month dosing at 4500 ppm, but corticosterone (53% of controls) and aldosterone (45% of controls) may have been depressed. All the above values in males and females returned to control values after the 8 weeks of recovery. The variation in estradiol values in males (66% of controls) and females (83% of controls) may have been due to artifacts of the limited number of animals used (4-10). If the values of estradiol did change in males and females, they apparently returned to control values after recovery. The basis of the determination of changes in these hormone values were non-overlapping standard deviations of the values, since no statistical analysis was conducted on hormone levels.

All females demonstrated cycles but all dosed females demonstrated abnormal estrous cycles. Control animals demonstrated 4 day cycles; two females in each control group demonstrated abnormal cycles, each characterized by various combinations of 2 and 11 days of continuous estrus and in two animals of the 4 animals, 2 days of continuous proestrus. The cycles in the dosed animals were short and long cycles (compared with 4 days) containing no diestrus or extra days of diestrus and some with no metestrus separated by 2 or 3 days of continuous estrus. One dosed recovery animal demonstrated 2 and 4 days of continuous estrus and 3 days of continuous proestrus. The effects on the estrous cycle would probably also affect female fertility, but effects on female fertility at 4500 ppm have not been directly studied.

Adrenal weights were elevated after dosing in males (288% of controls) and females (192% of controls), but returned to control values after recovery. Liver weights were nominally elevated in males (106% of controls, $p \geq 0.05$), but statistically significantly elevated in females (164% of controls, $p \leq 0.0001$). Male liver weights returned to control values after recovery, but female liver weights remained significantly elevated at a lower value (115% of controls, $p \leq 0.001$) after untreated recovery.

Cytochrome P450 was elevated in males (209% of controls) and females (181% of controls) after dosing, but both returned to control values after recovery.

Urine volumes and urinary ketosteroids were not reliable due to technical problems and were discounted by the registrant.

However, the hormone metabolite data (urinary ketosteroids) did not show treatment related effects after 3 months of dosing or 8 weeks of untreated recovery.

In males (85% of controls, $p \leq 0.001$) and females (98% of controls, $p \geq 0.05$), body weights after 3 months of dosing were less than controls and food consumption was reduced in males and females, but the relative efficiency of food utilization remained unchanged. Body weight gain was reduced in males (65% of controls) and females (94% of controls).

Core classification: Acceptable. This special study was adequately conducted, well reported and is acceptable. No guideline is available for the study.

MRID# 427061-01:

(2) Knuppen, R (January 1993) Translation: Final Report-Study of Possible Binding the Androgen Receptor in the Cytosol from a Cell Line Expressing the Androgen Receptor and from the Prostate tissue of the Rat. Reg. Doc. No. BASF 93/10058, Project No.: 21B0375/880930. Study conducted by the Institute of Biochemical Endocrinology, Lubreck Medical University, Germany for BASF. MRID# 427061-01.

CONCLUSIONS: This report will be reviewed at a later date if and when sufficient information becomes available to quantify the substances used in the binding studies and to correlated these affinities with the effects of vinclozolin/active metabolite(s) on the intact animal.

SUMMARY OF DATA SUBMITTED: The binding of various compounds (vinclozolin, mibolerone and flutamide) to rat prostate receptors, an androgen dependent mammary carcinoma MCF-Cell line, two cell lines from Leydig or Sertoli Cells of the mouse (TM-3 and TM-4) and one human prostate cell line (LNCAP) was determined. The relative binding affinities of these compounds were also studied.

The results of these studies were presented, but details of the methodology were omitted. The concentration of the receptors in rat prostate tissue and for human LNCAP cell lines were presented. The relative binding affinities of the above compounds and in various combinations were determined and reported along with the bases from the graphical representation. Unfortunately the summary results were not identified with the 21 graphical representations of the data.

Although, many relative binding affinities were presented, these affinities are not useable because the actual substances being bound were not determined. Kelce et al. (1994) has found that the parent compound, vinclozolin is probably not bound to androgen receptors; a metabolite/degradation product(s) have the highest binding affinities and that the metabolites/degradation product(s) should be stabilized prior to the determination. Like

wise the hydroxyflutamide metabolite of flutamide is the active binding agent to the androgen receptor. Without an indication of the degree of metabolic activation and conditions of binding (pH, etc.), the results of the vinclozolin-androgen receptor binding are marginally relevant, since the concentration of the substance binding is unknown and may be considerably different under other conditions.

Even with sufficient information on the study conditions used, the relevancy of those conditions to those found in the intact animal (and probably to the intact human) during a study maybe difficult to correlate for substances similar to vinclozolin. The main function of the androgen binding studies was to detect androgen binding of vinclozolin/product(s) to androgen receptors in order to help determine a probable mechanistic hypothesis for the vinclozolin effects produced. This has been accomplished by these data and confirmed by the data of Kelce et al. (1994).

The relevancy of the data will be determined when and if sufficient information becomes available. At this stage, no additional data on the binding affinities of vinclozolin or its metabolites appear warranted.

References:

Kelce, WR, E Monosson,MP Gamcisk, S Laws and LE Gray, Jr (1994) Environmental Hormone Disruptors: Evidence that Vinclozolin Developmental Toxicity is Mediated by Antiandrogenic Metabolites. Toxicology and Applied Pharmacology. 126, 276-285.

Primary reviewer: David G Anderson, PhD. David G Anderson 9/1/94.
Section 3, Tox. Branch 1 (H7509C).
Secondary reviewer: Karen Hamernik, PhD. KH 9/1/94
Section 3, Tox. Branch 1 (H7509C).

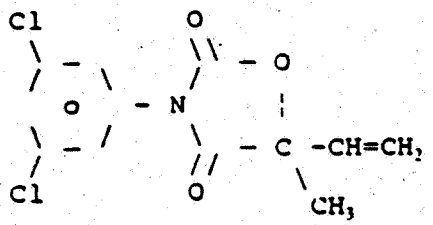
DATA EVALUATION REPORT

STUDY TYPE: Special 90-Day Study on Hormone Levels and Reversibility/92/11548/427061-02.

ToxChem No.: 323C. Submission No.: S443224.
PC No.: 113201. Case No.: 011409.
DPBarcode No.: D192566. MRID No.: 427061-02.

TEST MATERIAL: Vinclozolin, technical; A.I. is [3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedi-2,4-one].

STRUCTURE:



SYNONYMS: Ronilan™ (41 to 50% vinclozolin), Curalan (Turf)™, Ornalin™, Reg no. 83 258.

SPONSOR: BASF Corp. Chemicals Div., Ag. Chem., PO Box 13528, Research Triangle Park, NC 27709-3528.

TESTING FACILITY: BASF Aktiengesellschaft, Dept. Toxicology, D-W 6700 Ludwigshafen, Germany.

STUDY NO.: 92/11548. Reg. Doc. No. BASF 92/11548 (For 427061-02).

REPORT TITLE: Report-Study on the Influence of Reg. No. 83 258 (Vinclozolin) on the Hormone Status of Wistar Rats Administration in the Diet for 3-Months and a Recovery Period of at least 8-Weeks in Satellite Animals.

AUTHOR(S): Dr. W Mellert.

REPORT ISSUED: December 9, 1992.

EXECUTIVE SUMMARY: In a special study (BASF, 1992, MRID# 427061-01), vinclozolin was administered in the feed to 10 Wistar rats per sex per group to two control groups, 1 for 3 months and the other for 3 months plus 8 weeks at 0 mg/kg/day and to two dose groups, 1 for 3 months at 4500 ppm (about 300 mg/kg/day) and the other for 3 months at 4500 ppm, followed by 8 weeks of untreated recovery.

Several parameters were determined in these groups. Plasma levels of ACTH, LH, FSH, testosterone, corticosterone,

aldosterone, DHEA and estradiol were determined in the two control groups and in the two dose groups. Cytochrome P450 was determined in the livers of the same respective groups of animals. Urine volumes, urinary ketosteroids and adrenal and liver organ weights were also determined in the same respective groups of animals. Estrous cycles were determined in the females of the 4 respective groups for the 3 to 4 weeks before termination.

Plasma was obtained from males and females by decapitation; females were in diestrus at the time of decapitation. ACTH (178% of controls), LH (940% of controls), FSH (215% of controls), testosterone (276% of controls), DHEA (189% of controls) and may be corticosterone (160% of controls) and aldosterone (154% of controls) in males were elevated after 3 months of dosing at 4500 ppm, but returned to control values during the 8 week recovery. ACTH (255% of controls) and LH (250% of controls) were elevated in females after 3 month dosing at 4500 ppm, but corticosterone (53% of controls) and aldosterone (45% of controls) may have been depressed. All the above values in males and females returned to control values after the 8 weeks of recovery. The variation in estradiol values in males (66% of controls) and females (83% of controls) may have been due to artifacts of the limited number of animals used (4-10). If the values of estradiol did change in males and females, they apparently returned to control values after recovery. The basis of the determination of changes in these hormone values were non-overlapping standard deviations of the values, since no statistical analysis was conducted on hormone levels.

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Adrenal weights were elevated after dosing in males (288% of controls) and females (192% of controls), but returned to control values after recovery. Liver weights were nominally elevated in males (106% of controls, $p \geq 0.05$), but statistically significantly elevated in females (164% of controls, $p \leq 0.0001$). Male liver weights returned to control values after recovery, but female liver weights remained significantly elevated at a lower value (115% of controls, $p \leq 0.001$) after untreated recovery.

Cytochrome P450 was elevated in males (209% of controls) and females (181% of controls) after dosing, but both returned to control values after recovery.

Urine volumes and urinary ketosteroids were not reliable due

to technical problems and were discounted by the registrant. However, the hormone metabolite data (urinary ketosteroids) did not show treatment related effects after 3 months of dosing or 8 weeks of untreated recovery.

In males (85% of controls, $p \leq 0.001$) and females (98% of controls, $p \geq 0.05$), body weights after 3 months of dosing were less than controls and food consumption was reduced in males and females, but the relative efficiency of food utilization remained unchanged. Body weight gain was reduced in males (65% of controls) and females (94% of controls).

Core classification: Acceptable. This special study was adequately conducted, well reported and is acceptable. No Guideline is available for the study.

A. MATERIALS:

1. Test material: Vinclozolin, Description: Solid; Batch No.: N 183; Purity - 99.2% a.i.

2. Test animals: Species: Rats, Strain: Wistar (Chbb = THOM(SPF)), Age: 5 weeks at study initiation, Weight: Males - 284 (264 - 306) g, Females - 190 (168 - 220) g at study initiation, Source: Karl Thomae GmbH, Biberach an der Riss, FRG. Animals were acclimatized for 23 days after receipt.

3. Environment: The animal room was maintained at 20 to 24° C; Relative humidity was 30-70%; Light:dark = 12:12, starting at 6:00 AM. Rooms were disinfected with Autex apparatus, fully automatic. Final disinfecting used formaldehyde and ammonia. Each week walls and floor were disinfected with 0.5% Mikro-Quat.

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B. STUDY DESIGN:

1. Animal Assignment - Animals were assigned randomly to each group according to body weight.

Test group	Conc. in diet (ppm)	Number of animals (males)		Animal No.
		Main study	Satellite	
0	0	10		1-10
0S	0		10	11-20
1	4500	10		21-30
1S	4500		10	31-40
Positive control 0.5% phenobarbital treated		4	4	81-84 89-92
		Number of animals (females)		
0	0	10		41-50
0S	0		10	51-60
1	4500	10		61-70
1S	4500		10	71-80
Positive control 0.5% phenobarbital treated		4	4	85-88 93-96

2. Study Purpose and Protocol - The study is designed to provide information on the hormone levels induced by the administration of vinclozolin and untreated recovery. Since adrenal hormones were to be determined, stress was minimized by using the same animal handler and animal training. Phenobarbital positive controls (0.5% in the drinking water for about 10, 11, 12 and 13 days to 2 sets of 4/sex) were used for comparison with possible vinclozolin induced enzymes and as a methods check.

Cycle Determination
Daily vaginal smears were obtained the 3-4 weeks prior to sacrifice, but during diestrus.

Hormone Analyses

Blood was collected from decapitated animals in EDTA-K2 and trasylol containing viles in an ice bath, centrifuged (0-4°C) and deep frozen at -80°C and shipped to R. Knappen for analysis under dry ice. The following hormone analyses were conducted on the plasma. Since insufficient plasma was obtained for all analyses, estradiol was not determined on plasma from all animals.

Males	Females
1. Luteinizing hormone (LH)	1. Luteinizing hormone (LH)
2. Follicle stimulating Hormone (FSH)	2. Follicle stimulating hormone (FSH)
3. ACTH	3. ACTH
4. Testosterone (Testo)	4. Testosterone (Testo)
5. Corticosterone (Cortico)	5. Corticosterone (Cortico)
6. Aldosterone (Aldo)	5. Aldosterone (Aldo)
7. Estradiol (E2)*	7. Estradiol (E2)*

* Estradiol was determined only when sufficient plasma was available.

Urine Collection

Urine was collected during dosing and recovery periods in metabolism cages; the volume and breakdown products of testosterone, corticosterone and estradiol were determined.

Organ Collection

Livers were collected at study termination, cooled and microsomal cell fractions prepared and frozen -80°C for Cytochrome P450 determination (See Appendix for methods copied from the submitted report). Adrenal glands and prostates were also collected for R Knappen. The weights of livers and adrenal were determined.

3. Diet preparation - Diet was prepared and stored at room temperature until used. Samples of the diet were collected at study initiation and termination for analysis. Homogeneity (Study# 71S0375/88026) and stability (Study# 53S0375/88025) have been determined satisfactorily in other studies.

4. Animals receive food and water ad libitum. Rats were fed Kliba maintenance diet rat/mouse/hamster GLP 343 meal supplied by Klingentalmühle AG, CH-4303 Kaiseraugst, Switzerland. Tap water was also supplied.

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5. Statistics - The data were evaluated statistically using the computer systems of the Department of Toxicology of BASF, Aktiengesellschaft.

Body weights, organ weights, hormone analyses, clinical examinations and P450 determinations were analyzed by two sided tests, Students t-test.

6. Data presented in the submitted report was quality assurance audited throughout the study and signed by H Fleig, Head of QA on 12/9/92.

C. **METHODS AND RESULTS:** (Methods were copied from the submitted report and are reproduced and presented in Appendix I. Lettered tables were constructed from data in the submitted report)

1. Observations - Animals were inspected daily for signs of toxicity and mortality.

Results - Toxicity - No dose related signs were observed during the study, except 4 females at 4500 ppm in group 1 and 6 females at 4500 ppm in group 1S developed cataracts.

Mortality (Survival) - No mortality occurred during the study.

2. Body Weight, Food and Water Consumption - Body weights and body weight gain were determined weekly during dosing and recovery.

Results Body weights were decreased in males (85% of controls, $p \leq 0.001$) and females (98% of controls, $p \geq 0.05$) during the main study but they were no longer statistical significantly decreased after recovery when compared with control values. Statistical analyses were not conducted on body weight gain, but they were decreased in both males (65% of controls) and females (94% of controls) in the main study, but not after recovery (Table A).

Food consumption was decreased in males and females, but no biologically significant changes occurred in the relative efficiency of food utilization in males or females.

Table A: Body weight gain, food consumption data and food efficiency calculations for the pre-mating period.

	Main study		Recovery		Main study		Recovery	
	Males	Males	Males	Males	Females	Females	Females	Females
Group	0 ppm	4500 ppm	0 ppm	4500 ppm	0 ppm	4500 ppm	0 ppm	4500 ppm
Mean body weight at termination	503.9	427.1***	531.4	502.3	281.1	274.8	290.6	294.1
% of controls		84.8%		94.5%		97.8%		101%
Mean body weight gain	219.2	142.7	247.1	218.6	91.7	85.8	96.6	105.0
% of controls		65.1%		88.5%		93.6%		109%

*** = Statistically significant at $p \leq 0.001$.

3. Estrous cycles Estrous cycles were determined for 3 to 4 weeks before sacrifice. Animals were sacrificed during diestrus, only, in order to avoid the cyclic LH and FSH elevations during proestrus and estrus.

Results - There were no normal estrous cycles in females dosed with 4500 ppm for 3 months (cycles were characterized by several occasions of 2 and 3 days of continuous estrus inter-dispersed with an apparently normal, short or long cycles), however they appeared to return to normal during the 8 week recovery period (Table B). The cycle length for the rats on study was 4 days with only 2/10 having abnormalities in the cycle in each of the 3 month controls and 3 month controls + 8 week recovery groups.

The animals with the abnormal cycles in the group dosed at 4500 ppm had higher LH levels (255% of controls) and ACTH levels (287% of controls). These cycles and hormone levels of these groups of animals recovered during the 8 week recovery period.

Table B: Estrus cycles of rats in controls and during dosing at 4500 ppm for 3 months and 8 weeks recovery. Cycles were studied 3 to 4 weeks.

Group	No. with 4 day estrus cycles/(total no. of animals)	No. animals with abnormal cycles (type)/total no. of animals, i.e., no. days of continuous estrus or proestrus/total no. of animals			
	In normal cycles	No. with 2 days of continuous estrus	No. with 3 days of continuous estrus	No. with 4 days of estrus	No. with other abnormalities in estrus cycles
3 month control	8/10	1/10			1/10
3 month control + 8 week control for recovery	8/10	2/10 (including 1 with 2 days of proestrus)			1/10 (11 days of continuous estrus)
3 month dosing with 4500 ppm	0/10	8/10	2/10		
3 month dosing with 4500 ppm - 8 weeks untreated recovery	8/10	1/10	NA	NA	1/10 (including 1 animal with 2 & 4 days of continuous estrus & 3 days of continuous proestrus

NA = Not applicable because animal had other abnormalities in cycle.

4. Hormone Levels: The hormone levels were determined on plasma collected at termination by decapitation (Table C). Statistical significance was not presented in the report on the hormone levels, however estimates of elevation or depression are presented below based on the overlap in standard deviations and conclusions presented in the submitted report. After the % of controls, the coefficient of variation (CV) is presented below; CV = (Standard deviation/value) X 100.

Results -

ACTH levels - In males, they were elevated 178% (CV=48%) of controls (CV=32%) after 3 months of dosing at 4500 ppm, but returned to normal after 8 weeks of untreated recovery.
In females, they were elevated 255% (CV=28%) of controls (CV=39%) after 3 month of dosing at 4500 ppm, but returned to normal after 8 weeks of untreated recovery.

LH levels - In males, they were elevated 940% (CV=27%) of contro (CV=55%) after 3 months of dosing at 4500 ppm, but r to normal after 8 weeks of untreated recovery.
In females, they were elevated 250% (CV=22%) of controls (CV=42%) after 3 month of dosing at 4500 ppm, but returned

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to normal after 8 weeks of untreated recovery.

FSH levels - In males, they were elevated 215% (CV=13%) of controls (CV=23%) after 3 months of dosing at 4500 ppm, but returned to normal after 8 weeks of untreated recovery.
In females, they did not change after 3 month of dosing at 4500 ppm and remained normal after 8 weeks of untreated recovery.

Testosterone levels - In males, they were elevated 276% (CV=41%) of controls (CV=34%) after 3 months of dosing at 4500 ppm, but returned to normal after 8 weeks of untreated recovery.
In females, they did not change after 3 month of dosing at 4500 ppm and remained normal after 8 weeks of untreated recovery.

Corticosterone levels - In males, they may have changed to 160% (CV=54%) of controls (CV=31%) after 3 months of dosing at 4500 ppm, but were essentially similar to controls after 8 weeks of untreated recovery.
In females, they may have changed to 53% (CV=36%) of controls (CV=38%) after 3 month of dosing at 4500 ppm, but were essentially similar to controls after 8 weeks of untreated recovery.

Aldosterone levels - In males, they may have changed to 154% (CV=42%) of controls (CV=33%) after 3 months of dosing at 4500 ppm, but were essentially similar to controls after 8 weeks of untreated recovery.
In females, they may have changed 45% (CV=58%) of controls (CV=47%) after 3 month of dosing at 4500 ppm, but were essentially similar to controls after 8 weeks of untreated recovery.

DHEA levels -In males, they appeared to change to 189% (CV=12%) of controls (CV=8%) after 3 months of dosing at 4500 ppm, but were essentially similar to controls after 8 weeks of untreated recovery.
In females, they remained normal after 3 month of dosing at 4500 ppm and remained normal after 8 weeks of recovery.

Estradiol (E2) levels - In males, they may have changed to 66% (CV=23%) of controls (CV=39%) after 3 months of dosing at 4500 ppm, but were essentially similar to controls after 8 weeks of untreated recovery.
In females they may have changed to 83% (CV=37%) of controls (CV=17%) after 3 month of dosing at 4500 ppm, but may have changed to 144% (CV=26%) of controls (CV=9%) after 8 weeks of untreated recovery.
The changes in estradiol levels in males and females may

have been caused by an artifact of the limited number of plasmas analyzed in the treated groups.

Table C: Hormone levels after treatment with 4500 ppm of vinclozolin for 3 months at termination and 8 weeks of recovery untreated in males and females.

	ACTH (pg/ml)	LH (ng/ml)	FSH (ng/ml)	Testo (ng/ml)	Cortico (ng/ml)	Aldo (ng/ml)	DHEA (ng/ml)	E2 (ng/ml)
Male hormone levels in controls, untreated for 3-months ± standard deviation (No. of animals).								
Values	98±31 (9)	0.42±0.23 (9)	9.5±2.2 (9)	2.9±1.0 (9)	236±73 (9)	426±137 (9)	0.75±0.06 (9)	8.7±3.4 (4)
Male hormone levels controls, untreated for 3-months + 8 weeks untreated ± standard deviation (No. of animals).								
Values	200±73 (10)	0.64±0.39 (10)	8.8±1.0 (10)	4.4±3.1 (10)	206±115 (10)	475±336 (10)	1.06±0.31 (10)	5.0±0.9 (5)
Male hormone levels, 4500 ppm treated for 3-months ± standard deviation (No. of animals).								
Values	274±83 (10)	3.95±1.07 (10)	20.4±2.6 (10)	8.0±3.3 (10)	381±207 (10)	669±283 (10)	1.42±0.17 (10)	5.7±1.3 (5)
Male hormone levels, 4500 ppm treated for 3-months + 8 weeks untreated ± standard deviation (No. of animals).								
Values	92±47 (10)	0.50±0.30 (10)	10.5±1.7 (10)	3.7±1.6 (10)	174±82 (10)	408±336 (10)	0.87±0.11 (10)	5.0±0.9 (5)
Female hormone levels in controls, untreated for 3-months ± standard deviation (No. of animals).								
Values	174±68 (10)	0.53±0.22 (10)	5.9±0.5 (10)	0.13±0.02 (10)	726±274 (10)	1110±518 (10)	0.79±0.15 (10)	15.4±2.6 (10)
Female hormone levels in controls, untreated for 3-months and 8 weeks ± standard deviation (No. of animals).								
Values	102±43 (10)	0.64±0.38 (10)	6.4±1.0 (10)	0.12±1.0 (10)	695±209 (10)	962±344 (10)	0.67±0.13 (10)	13.3±1.2 (10)
Female hormone levels, 4500 ppm treated for 3-months ± standard deviation (No. of animals).								
Values	499±142 (10)	1.35±0.30 (10)	5.7±0.7 (10)	0.13±0.03	383±137 (10)	503±230 (10)	0.78±0.13 (10)	12.8±4.7 (4)
Female hormone levels, 4500 ppm treated for 3-months + 8 weeks untreated ± standard deviation (No. of animals).								
Values	261±95 (10)	0.71±0.22 (10)	6.6±0.7 (10)	0.14±0.01 (10)	611±95 (10)	1179±423 (10)	0.82±011 (10)	19.1±5.0 (4)

5. Cytochrome P450, urine volume, ketosteroids, adrenal weight and liver weight.

Results - Cytochrome P450 appeared to elevated in males (210% of controls) and in females (181% of controls) during the 3 months dosing at 4500 ppm and both return to normal during the recovery period (Table D).

Urine volumes were considered not to be sufficiently accurate to determine differences, but may have been elevated in females (236% of controls) during the 3 month dosing with 4500 ppm (Table D).

Due to technical errors of unspecified nature, the registrant discounted the meaning of the urinary ketosteroid determinations.

Adrenal weights were elevated in males (288% of controls) and in females (192% of controls) during dosing, but returned to normal after recovery (neither were statistically significant).

Liver weights were elevated (164% of controls, $p \leq 0.001$) in females, only, after the dosing period and were still elevated (116% of controls, $p \leq 0.001$) at the end of recovery (Table D).

Table D: Cytochrome P450 in liver, urine volume and urinary ketosteroids levels after treatment with 4500 ppm of vinclozolin for 3 months at termination and 8 weeks of recovery untreated in males and females.

	Cytochrome P450 (nmol/mg protein)	Urine volume (ml)	Keto-steroids (µg/day)	Adrenal Wt. (g)	Liver Wt. (g)
Male levels in controls, untreated for 3-months ± standard deviation (No. of animals).					
Values	0.195±0.028 (5)	6.7±1.8 (9)	27±10 (9)	0.0673±0.0076 (9)	20.41±2.48 (9)
Male levels controls, untreated for 3-months + 8 weeks untreated ± standard deviation (No. of animals).					
Values	0.226±0.066 (10)	4.5±1.0 (10)	34±10 (10)	0.0620±0.0066 (10)	19.32±1.60 (10)
Male levels, 4500 ppm treated for 3-months ± standard deviation (No. of animals).					
Values	0.409±0.099 (6)	7.5±2.5 (10)	48±22 (10)	0.1941±0.0457 (10)	21.61±2.50 (10)
Male levels, 4500 ppm treated for 3-months + 8 weeks untreated ± standard deviation (No. of animals).					
Values	0.178±0.036 (10)	5.2±1.3 (10)	31±10 (10)	0.0771±0.0125 (10)	19.37±3.15 (10)
Female levels in controls, untreated for 3-months ± standard deviation (No. of animals).					
Values	0.140±0.034 (5)	2.5±0.4 (10)	46±14 (10)	0.0832±0.0064 (10)	10.30±1.13 (10)
Female levels in controls, untreated for 3-months and 8 weeks ± standard deviation (No. of animals).					
Values	0.201±0.043 (10)	3.1±1.0 (10)	38±12 (10)	0.0875±0.0104 (10)	10.23±0.91 (10)
Female levels, 4500 ppm treated for 3-months ± standard deviation (No. of animals).					
Values	0.254±0.075 (3)	5.3±1.3 (10)	54±16 (10)	0.1597±0.0278 (10)	16.9±.90***(10)
Female levels, 4500 ppm treated for 3-months + 8 weeks untreated ± standard deviation (No. of animals).					
Values	0.169±0.059(10)	4.2±0.8 (10)	58±13 (10)	0.0841±0.0147 (10)	11.8±1.36**(10)

* = Statistically significant at $p \leq 0.05$.; ** = Statistically significant at $p \leq 0.01$; *** = Statistically significant at $p \leq 0.001$.

6. Cytochrome P450 levels in the phenobarbital positive controls

The registrant discounted the meaning of the studies on these limited number of animals. However, the cytochrome P450 levels appeared to be higher in treated groups than in the recovery groups. Apparently the control animals used were the untreated rats from the vinclozolin segment of the cytochrome P450 analyses (Table E). The results from all animals are not reported because of technical problems.

Table E: Cytochrome P450 in liver tissue in controls and in 0.5% sodium phenobarbital treated males and females for 10, 11, 12 and 13 days (means of another 8 animals treated similarly and the allowed to recover untreated).

Male cytochrome P450 levels (nmol/mg protein) in 0.5% phenobarbital treated group ± standard deviation (No. of animals included in the average).81-84	Recovery male rats cytochrome P450 levels (nmol/mg protein) in 0.5% phenobarbital treated group ± standard deviation. Recovery rats (No. of animals included in the average).89-92	Female cytochrome P450 levels (nmol/mg protein) in 0.5% phenobarbital treated group ± standard deviation. (No. of animals included in the average).85-88	Recovery female rat cytochrome P450 levels (nmol/mg protein) in 0.5% phenobarbital treated group ± standard deviation (No. of animals included in the average).93-96
0.784±0.088 (2)	0.554±0.195 (4)	0.311±0.013 (2)	0.231±0.019 (4)

Male cytochrome P450 levels (nmol/mg protein) in controls ± standard deviation (No. of animals).

G. APPENDIX I:

Moreover the adrenal glands were removed and weighed from all animals (with the exception of positive control animals) and the prostate from the male control animals on the day of sacrifice and were frozen for transport at -80°C, sent to Prof. Dr. R. Knuppen under dry ice cooling.

After sacrifice the weight of the exsanguinated animals, all livers and adrenal glands were determined.

3.8.5. Liver tissue processing

The liver was removed from each animal, weighed, and placed into ice-cold 50 mM-Tris buffer, pH 7.4, containing 0.25 M-sucrose.

All subsequent operations were conducted in an environment maintained at 4°C.

Livers were homogenised individually in about 4 volumes 50 mM Tris buffer, pH 7.4, containing 0.25 M-sucrose using an electrically-driven glass Potter-Elvehjem-type homogeniser, fitted with a Teflon pestle. The homogenates were centrifuged at 9,000 g for 20 minutes at 4°C (Beckmann Ultracentrifuge), and the resulting supernatant was decanted. Microsomal fractions were prepared by centrifugation of this supernatant at 100,000 g for 1 hour at 4°C (Beckmann Ultracentrifuge). The supernatant was decanted. The microsomal pellet was suspended in about 5 - 6 ml, 50 mM Tris buffer, pH 7.4. The microsomal suspension was centrifuged a second time at 100,000 g for 1 hour at 4°C and the pellet stored at -80°C. The determination of cytochrome P_{450} was performed on the day after the microsome preparation.

The remaining microsomal suspension was stored at -20°C until required for protein analyses.

3.8.6. Measurement of microsomal protein

Protein concentration was determined by the method of LOWRY et al. (1951). Aliquots (0.5 ml in duplicate) of microsomal suspension were diluted in 9 ml aqua bidest. and 0.5 ml 0.1 N NaOH and mixed with alkaline copper tartrate reagent [1 part 2% (w/v) Na/K Tartrate : 1 part 1% (w/v) $CuSO_4$ + 100 parts 2% (w/v) Na_2CO_3 in 0.1 N NaOH] (5 ml) and left at room temperature for 10 minutes. Aliquots (0.5 ml) of Folin-Ciocalteus phenol reagent (diluted 1 : 1 with water) were then added with immediate mixing. The mixture was left at room temperature for a further 30 minutes. Samples were assayed spectrophoto-

metrically at 750 nm against a distilled water blank, and the amount of protein calculated from a standard curve prepared from 0 to 200 µg bovine serum albumin assayed under identical conditions.

3.8.7. Measurement of cytochrome P_{450}

Cytochromes P_{450} was assayed by the method of OMURA and SATO (1967). Microsomal suspension (1 ml) was mixed with 50 mM-Tris buffer, pH 7.4, (1.0 ml) in two cuvettes. A base-line spectrum (415 to 490 nm) was taken to ascertain that the content of both cuvettes was identical.

To each of the cuvettes 0.02 ml of an aqueous sodium dithionite solution (200 mg/ml) was added. After mixing carbon monoxide was bubbled through the sample cuvette for 1 minute, and the absorption difference spectrum from 490 to 415 nm was recorded. Cytochrome P_{450} concentrations were calculated from the optical density difference (450 to 490 nm) using the molar extinction coefficient of 91 nM cm^{-1} and the protein concentration according to the following formula:

[(ABS 450 - ABS 490 nm) : 91] : (Protein content x 0,495) = µmol P-450 per mg Protein

ABS = Absorption

91 = Extinction factor $\left[\frac{\mu M \text{ p-450}}{cm}\right]$

Protein content (mg/ml)

0,495 = Dilution factor

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