ANDROLOGIA **32,** 285–293 (2000)

Male adolescent exposure to endocrine-disrupting pesticides: vinclozolin exposure in peripubertal rabbits

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Key words. Accessory sex gland—anti-androgenic—dermal dosing—metabolic products

Summary. Adolescence is a time of dramatic neuroendocrine changes that are required for sexual maturation. Hormonal mimicking or inhibiting chemicals can cause significant impairment during this critical period. Vinclozolin (Vin) has been shown to be an anti-androgen affecting male offspring in rats *in utero*, and its mechanism of action may be mediated by inhibition of androgenic receptor action. The majority of teenagers working on farms are male, and therefore a systemic fungicide, vinclozolin, was selected for study. The rabbit has proved to be an excellent species for modelling reproductive toxicant effects in the male and was selected as the test species. The peripubertal phase for the rabbit was determined to be between the 3rd and 4th months. A 2-month dosing period was therefore initiated at 3 months of age and carried through to the 4th month. Vin was administered by dermal application ($100\ mg\ kg^{-1}$ in $100\ \mu l$ of dimethylsulphoxide) daily. Body weights were determined weekly. The rabbits were then held until fully mature (6 months of age). Semen was collected and evaluated from sexually mature males on a weekly schedule for 5 weeks to maximize sperm output. An automated solid phase extraction procedure for monitoring exposures through isolation and quantification of Vin and its metabolic products was developed. Increased plasma levels of Vin and M2 were found throughout the experimental period. The exposed rabbits had a smaller weight gain during pubertal growth (approaching significance; $P=0.059$). At maturity, the accessory sex glands of the exposed animals weighed less than those of the controls ($P=0.016$). Surprisingly, the pooled sperm count of the exposed animals was significantly higher ($P=0.017$) than that of the unexposed animals. The anti-androgenic effects of Vin may have blocked the negative feedback mechanism of testosterone on the hypothalamus or pituitary gland, allowing for an increase in gonadotrophin release, and consequently increasing sperm production at puberty.

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Introduction

In the 1996 publication of the book *Our Stolen Future* (Coulborn *et al.*, 1996), the hypothesis is presented that many chemicals currently in commerce interfere with normal endocrine activity causing adverse human health effects. The book is written in the style of a scientific mystery, including both scientific research and speculation, and outlines a public health threat to the general populus. This idea, however, is not a new one. There was much research on the effects of environmental chemicals disrupting the reproductive endocrinology of wildlife [e.g. effects of dichlorodiphenyltrichloroethane (DDT)] in the 1960s. The difference between the earlier research and that appearing in 1996 was that recent, highly publicized scientific reports suggested chemical effects on more than just otters and sea gulls—it was feared that the endocrine-active chemicals may affect human health, and in the very sensitive areas of reproductive development in children and consequently reproductive function in adults. A 1992 report on declining sperms counts (Carlsen *et al.*, 1992), the Sharpe–Skakkebaek hypothesis (Sharpe & Skakkebaek, 1993) linking exogenous oestrogens to falling sperm counts, wide publicity about the cancers that developed from *in utero*

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exposure to diethylstilboestrol and a basic understanding of the effects of exogenous hormones from oral contraceptives on reproduction combined to create a general population ready, willing and able to comprehend the premise and hypothesis of *Our Stolen Future*. Public concern about this health issue generated several responses from the government. The preface of *Our Stolen Future* was written by Vice President Gore, and the United States Congress passed legislation requiring the US Environmental Protection Agency to develop a screening and testing strategy for chemicals that are endocrine disruptors. The National Institute for Occupational Safety and Health (NIOSH) has responded to these health issues by supporting research to evaluate potential health effects in workers exposed to endocrine-disrupting chemicals. Most of this research is involved in assessing the effects of endocrine active chemicals and is being conducted by the National Toxicology Program and the U.S. Environmental Protection Agency (EPA). Recent studies conducted by the EPA have revealed the anti-androgenic effects of Vin, a dicarboximide fungicide. They found a pattern of malformations similar to the anti-androgen flutamide. A follow-up study with Vin demonstrated that dose levels of 100 mg kg^{-1} day^{-1} delayed the development of androgen-dependent tissues and altered androgen receptor function. Adolescence is a time of dramatic neuroendocrine shifts; therefore, hormonal mimicking or inhibiting chemicals may cause a disproportionate alteration in normal sexual maturation.

A substantial number of the xenobiotics identified as endocrine disrupters are pesticides which cause a disproportionate alteration in normal sexual development (Sonnenschein & Soto, 1998). Seven per cent of all hired farmworkers are between the ages of 14 and 17. In addition, 1.2 million children under the age of 17 live on US farms and ranches (Committee on the Health and Safety Implications on Child Labor, p. 144, 1998). A 1997 report indicated that 38% of teenagers working on farms in North Carolina used pesticides or other agrochemicals (Shulman *et al.*, 1997). This does not include those teenagers who are exposed by over-spray and contaminated homes and work sites. Another study of migrant farmworkers under the age of 18 found that 10% prepared or mixed pesticides using only cloth gloves for safety equipment. Forty per cent reported working in fields still wet with pesticides (Committee on the Health and Safety Implications on Child Labor, p. 84, 1998).

The majority of teenagers working on farms are male, and therefore a widely used pesticide that may affect male sexual development was selected for study. A systemic fungicide, vinclozolin (Vin) CAS 50471-44-8 [3-(3,5-dichlorophenyl)-5-methyl-vinyloxazolidine-2,4-dione], has been used on fruits and vegetables along both the east and west coasts and the upper mid-west of the USA (Fig. 1). It has been shown to be an anti-androgen affecting male offspring in rats *in utero* (Gray *et al.*, 1994), and its mechanism of action may be mediated by inhibition of androgenic receptor action (Kelce *et al.*, 1994; 1997).

Materials and methods

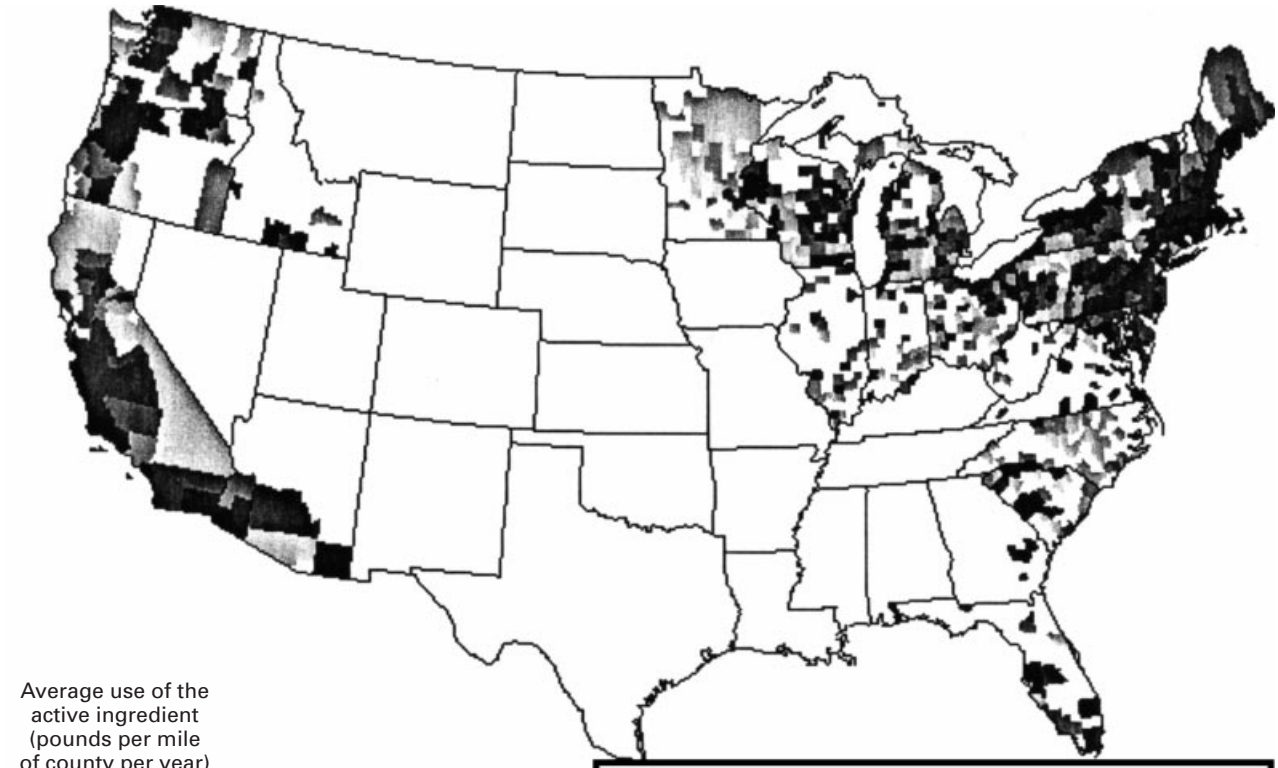
Important criteria for an animal model of human male reproduction must include the existence of documented, relatable physiology for the species and the ability to analyse ejaculated semen. Rabbits are the smallest common laboratory species from which serial semen samples can be readily obtained for functional, morphological, biochemical and quantitative fertility assessment. The rabbit has proved to be an excellent species for modelling reproductive toxicant effects in the male (Williams *et al.*, 1990; 1991; Foote *et al.*, 1995; Moorman *et al.*, 1998) and was therefore selected as the test species.

Exposure assessment

An automated solid phase extraction (SPE) procedure for monitoring short-term exposures through the rapid isolation and quantification of Vin and its metabolic products from plasma was developed (Fig. 2). Plasma samples and tissue filtrates were processed using a BenchMate® robotic workstation (Zymark, Hopkington, MA). The procedure developed for isolation and clean-up of the plasma products utilized Varian C8 reverse-phase SPE columns. Elution of products of interest was evaluated and optimized for percentage recovery as well as rapid sample preparation and analysis. Acetone eluates were analysed (Fig. 3) on an EI Hewlett-Packard quadrupole gas chromatograph-mass spectrometer (GC-MS) in the selected ion mode (SIM).

Synthesis of vinclozolin metabolites

3,5-dichloroaniline (M3, CAS 626-43-7) was purchased from Aldrich Chemical Co (Milwaukee, WI). Synthesis of the major metabolites of Vin, [2-[[(3,5-dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butanoic acid (M1, CAS 119209-27-7)



Crops	Total pounds applied	Per cent national use
Lettuce	50820	42.44
Green beans	23436	19.57
Strawberries	21688	18.11
Peaches	13598	11.38
Raspberries	3804	3.18
Cherries	2786	2.33
Nectarines	1161	0.97
Plums	844	0.70
Apricots	828	0.69
Onions	622	0.52

Figure 1. Estimated annual agricultural use of vinclozolin in the United States. United States Geological Survey, Pesticide National Synthesis Project.

and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2, CAS 83792-61-7)] was conducted as described by Szeto *et al.* (1989b). Vin (0.5 g, 1.7 mM) was incubated in 1 l of 0.1 M phosphate buffer (pH 8) for 2 weeks at 60 °C. The reaction mix was cooled and metabolite M2 was extracted with dichloromethane. After acidification to pH 1, M1 was extracted as above. The fractions were dried over Na_2SO_4 and concentrated *in vacuuo* for isolation by high-pressure liquid chromatography (HPLC).

HPLC analysis was conducted using a Hewlett-Packard 1090M HPLC system and a water : methanol gradient using a 4.6 mm by 25 cm Supelco LC-18S column (Supelco). Vin metabolites were isolated and evaluated for purity using a 20–100% linear methanol gradient in 1% acetic acid. Metabolites were dried *in vacuuo* for use as GC-MS standards. In addition to GC-MS identification of compounds, confirmation of compound identities was performed using the same column and separation conditions on a Finnigan LCQ HPLC/tandem mass spectrometer with electrospray ionization. The mass spectrometer was operated in the positive ion MS–MS spectrometric mode.

Sample preparation

Skin from dermal application sites and testes was excised at termination, homogenized and filtered. Analysis of plasma samples and calibration standards was conducted after SPE sample preparation using a BenchMate® robot workstation (Zymark).

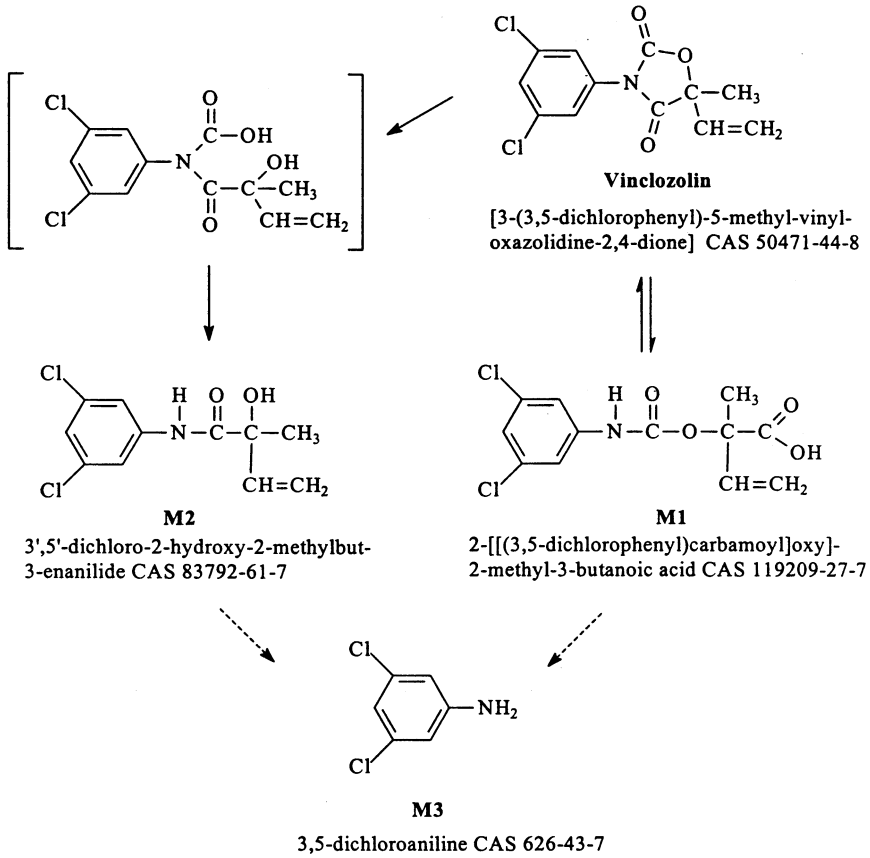


Figure 2. Pathways for metabolism of vinclozozin. Structural formulae of the parent and metabolites.

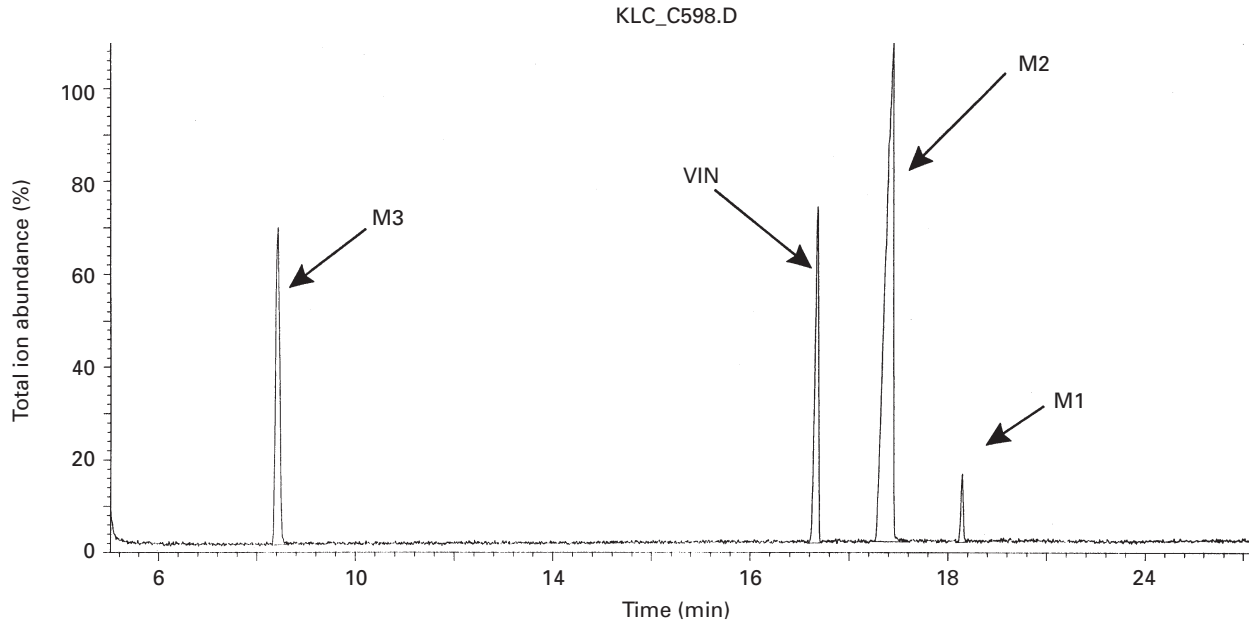


Figure 3. Single ion GC-MS chromatogram of vinclozolin and metabolites M1, M2 and M3.

The individual plasma samples and mixed standard compounds were applied to the Varian C8 SPE columns, washed and concentrated using a step-wise procedure (Table 1). The samples were stored at −20 °C until analysis by GC-MS.

GC-MS analysis was conducted using a Hewlett-Packard 5890A gas chromatograph and a 5970B quadrupole mass spectrometer, operated in either the scanning or the SIM mode with an accelerating voltage of 70 eV and a 1-ml min^{-1} helium carrier

Table 1. The Zymark BenchMate® II Workstation vinclozolin procedure: stepwise sample preparation of rabbit plasma samples using the automated system

Prepare rabbit plasma—aliquot 3 ml into sample tube
Initiate Zymark BenchMate® II software program which will:
Add 3 ml H_2O, pH 7
Vortex sample
Determine sample density
Condition Varian C8 SPE column with 3 ml methanol
Condition Varian C8 SPE column with 3 ml H_2O, pH 7
Load sample onto column
Rinse column with 3 ml H_2O, pH 7
Repeat rinse column with 3 ml H_2O, pH 7
Collect 1.5 ml fraction using acetone
Dry sample under N_2 using Zymark Turbo Vap LV Evaporator at 30 °C
Redissolve sample in either 1 ml or 100 µl acetone for GC-MS

gas flow. Samples of plasma or tissue extracts were analysed using a 75–250 °C thermal gradient on a 12 m by 0.2 mm internal diameter Hewlett-Packard Ultra 2 capillary column. Compounds of interest had fragmentation patterns consistent with those reported previously (Szeto *et al.*, 1989b). For both selectivity and sensitivity the SIM mode analysis was used. The major ions at m/z = 71, 72, 161, 174, 187, 188, 212, 214, 219 and 287, were used for quantification of Vin, M1, M2 and M3, using external calibration with appropriate standards. Calibration curves were linear with LOQs for Vin, M1, M2 and M3 at 50 ng ml^{-1}, 0.1 µg ml^{-1}, 1 µg ml^{-1} and 1 µg ml^{-1}, respectively.

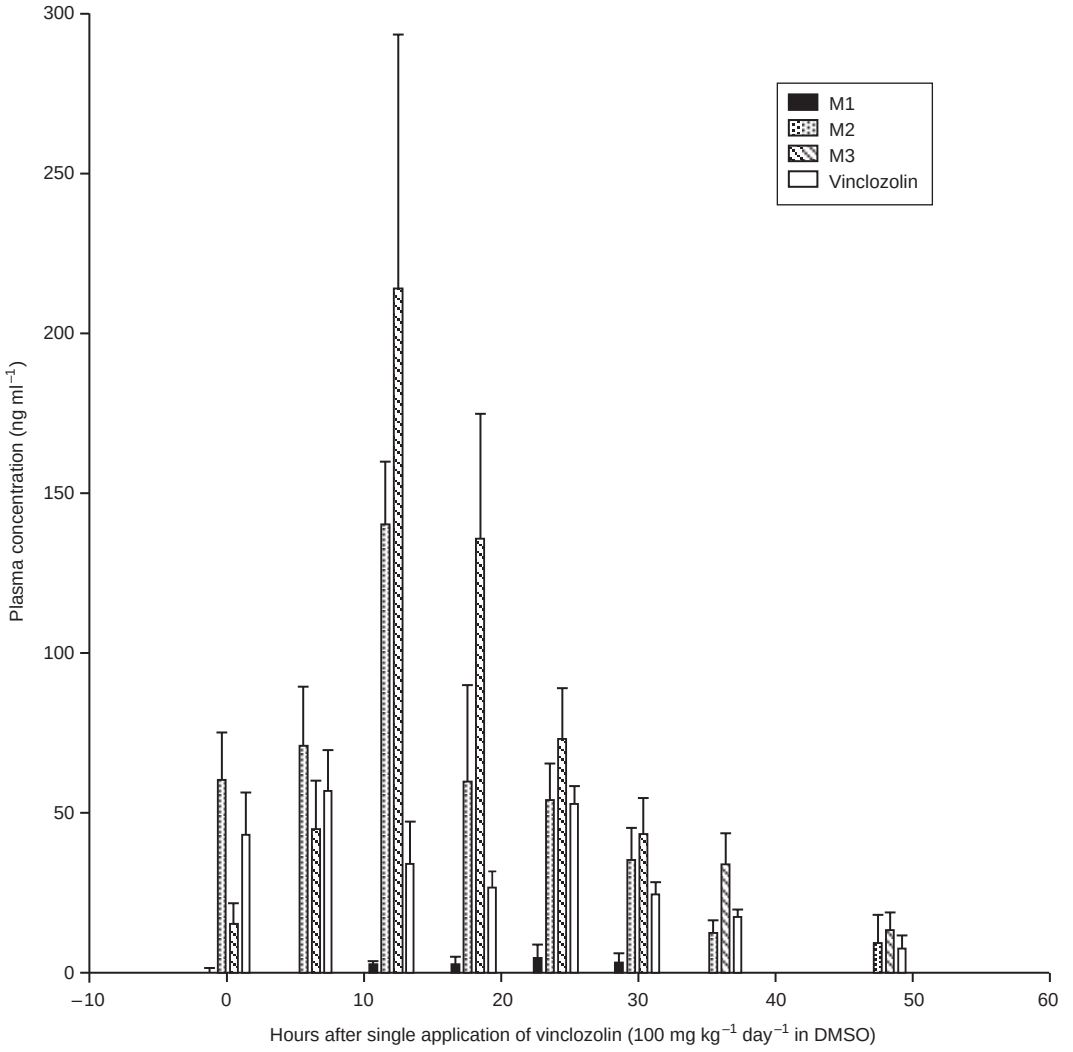


Figure 4. Plasma levels of vinclozolin and metabolites: Effect of time on plasma levels after a single dermal dose of vinclozolin (100 mg per kg body weight).

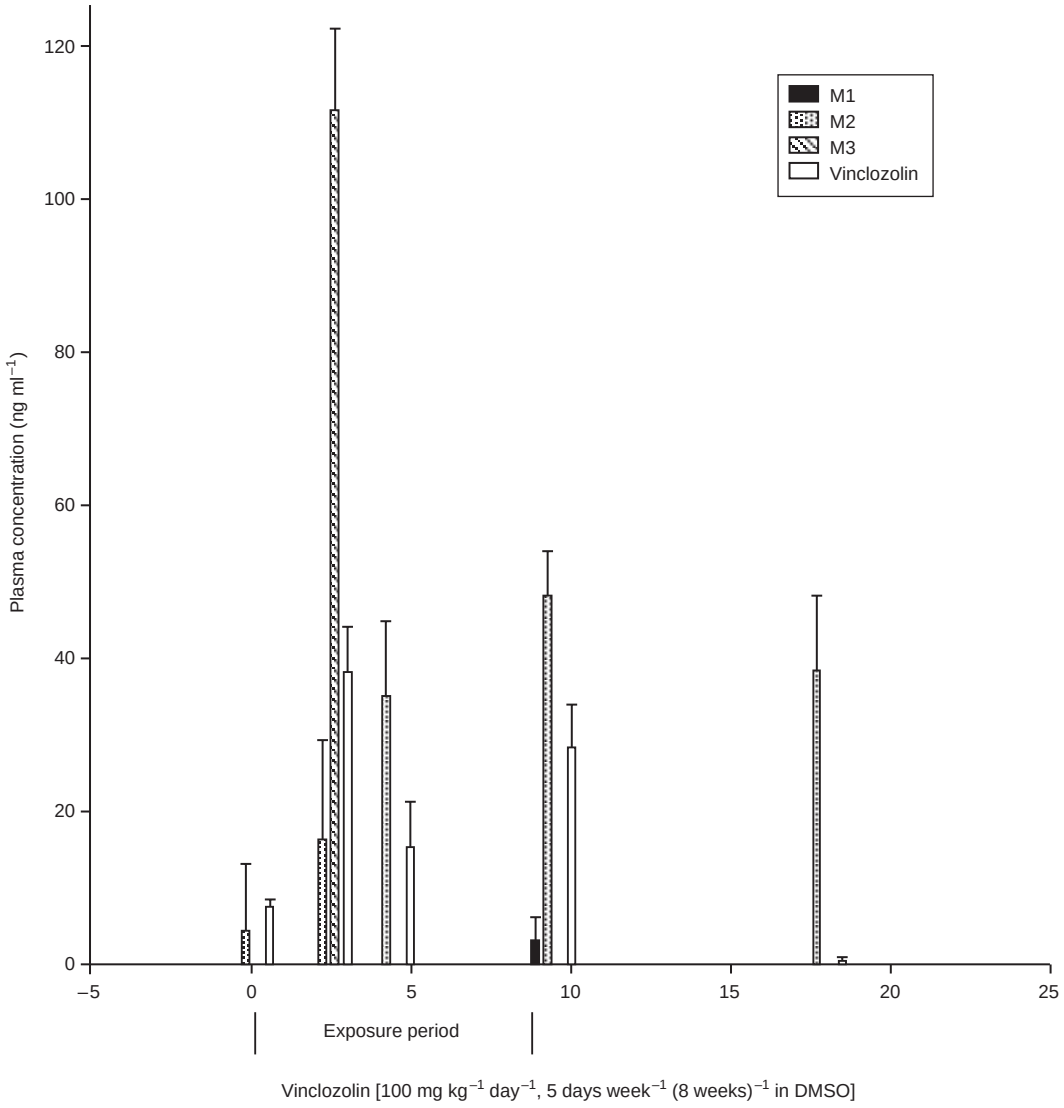


Figure 5. Plasma levels of vinclozolin and metabolites. Effect of time on plasma levels after multi-dose dermal administration of vinclozolin at 100 mg kg^{-1} per day, 5 days per week for 8 weeks. The animals were killed 10 weeks after the last vinclozolin dose.

Dose determination

In order to determine the dosing schedule and an effective biomarker of exposure, a pilot study was conducted. Five Dutch Belted rabbits were administered Vin by dermal application [100 mg kg^{-1} in dimethylsulphoxide (DMSO)]. DMSO facilitated extraction of the commercial grade Vin and facilitated dermal absorption. Plasma samples were taken at 0, 6, 8, 12, 24 and 48 h after a single dose. Following a single dermal administration of Vin, plasma levels of Vin and its metabolites peaked at 10 h. Vin, M2 and M3 were detectable after 50 h (Fig. 4).

Vinclozolin exposure to peripubertal rabbits

Upon receipt from the supplier, 2½-month-old (juvenile/adolescent) Dutch Belted rabbits underwent standard rabbit quarantine and were ear-tagged for identification. The peripubertal phase for the rabbit was determined to be between the 3rd and 4th months (Gondos, 1980). A 2-month dermal dosing period was therefore initiated at 3 months of age and carried through to the 4th month. Vin was administered by dermal application (100 mg kg^{-1} in 100 µl of DMSO) daily, Monday to Friday, throughout the 2-month dosing period which was completed as the rabbits reached 5 months of age. The control rabbits received a dermal application of DMSO without Vin. Body weights were determined weekly. The rabbits were then held until fully mature (6 months of age). Semen was collected from sexually prepared (three false mounts) mature males on a regular, once a week, schedule for 5 weeks to maximize sperm output. Each male ejaculated four times with a 20–30-min rest between collections. The

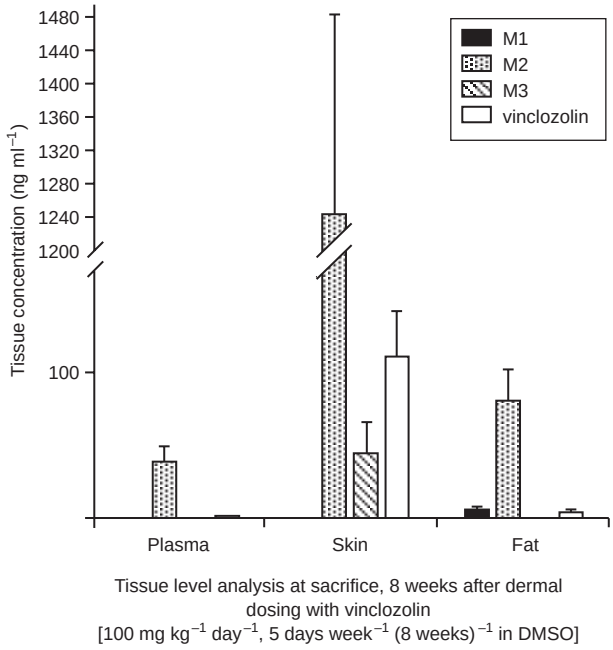


Figure 6. Tissue levels of vinclozolin and metabolites. Male rabbits were administered multiple doses of vinclozolin at 100 mg kg^{-1} per day, 5 days per week for 8 weeks by the dermal route. Tissues were harvested at killing 10 weeks after the last vinclozolin dose.

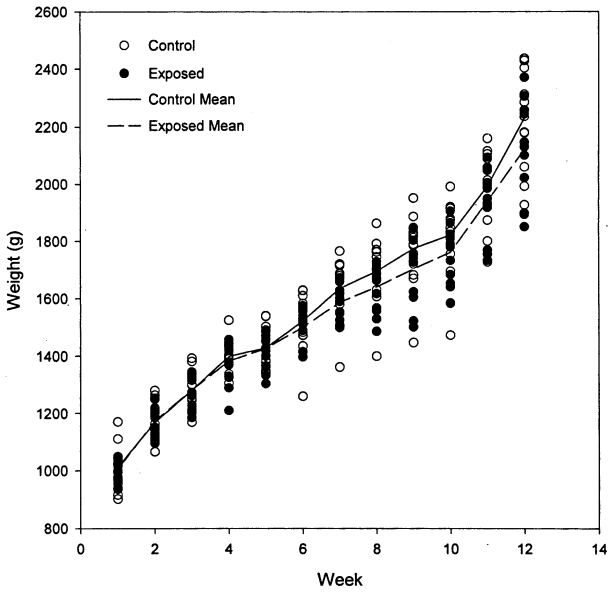


Figure 7. Scatter diagram of individual rabbit weights. The lines represent the mean weights for the control and exposed groups.

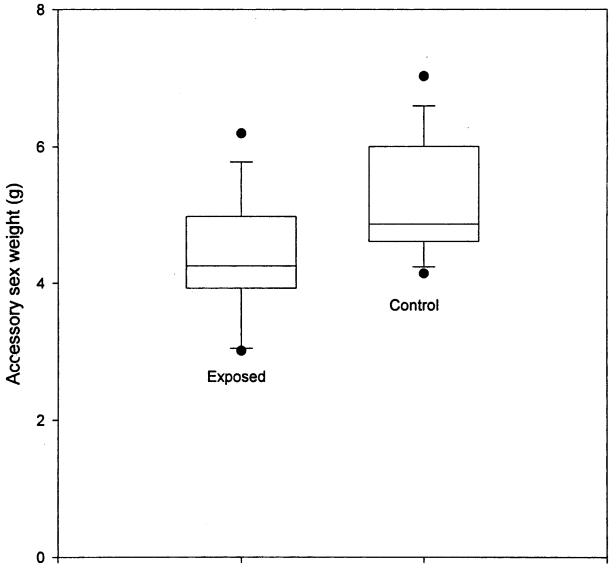


Figure 8. Box plot of accessory sex gland weights. This plot shows outlier data for both the exposed and control groups.

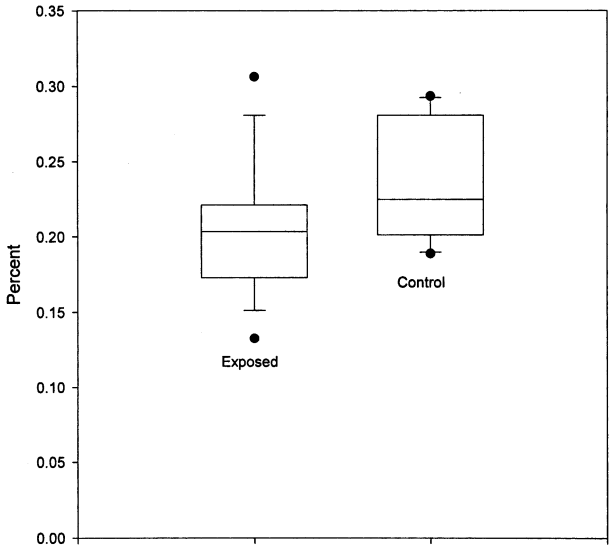


Figure 9. Box plot of the per cent of accessory sex gland weights to total rabbit weight. This plot shows outlier data for both the exposed and control groups.

procedure for each collection involved introducing the female (teaser doe) into the male's cage. Following three attempted mounts, the female was held with an artificial vagina situated such that the ejaculated semen was collected in the artificial vagina. Semen was analysed using an automated Hamilton-Thorne IVOS system.

Blood was collected (5 ml plasma and 1.5 ml of serum) every other week for analysis of blood levels of the reproductive hormones, as well as Vin and its major metabolites. Blood collection was conducted during the 2-month dosing period and once at termination.

At termination animals were weighed and then killed by overdose with a concentrated solution of sodium pentobarbital (approximately 75 mg kg^{-1}) via the marginal ear vein. The abdominal cavity was opened via midline incision. Both testes were measured and weighed. One testis was frozen (-80 °C) for analysis of Vin levels. The accessory sex organs were dissected from the body as a single unit and weighed. The rabbits were examined for gross evidence of intercurrent disease.

Statistics were conducted by The Mixed

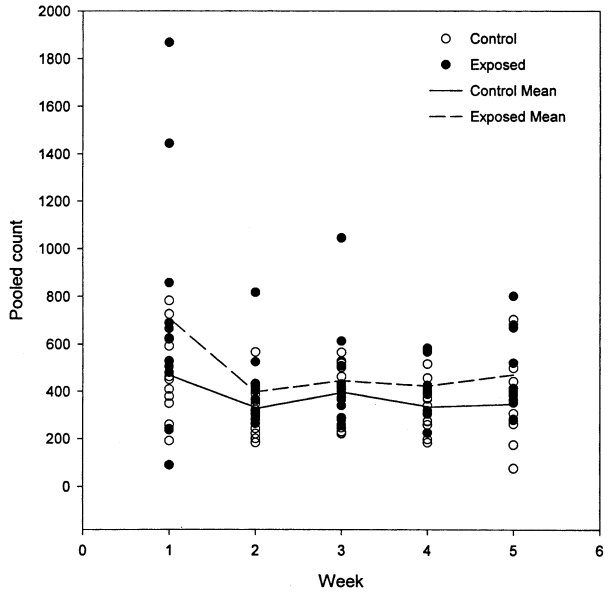


Figure 10. Scatter diagram of individual rabbit pooled sperm counts. The lines represent the mean pooled sperm counts for the control and exposed groups.

Procedure (REML Estimation Iteration History) using SAS. Only the last 4 weeks of the 5-week period of semen collection and analysis were tested statistically because a large difference (both control and treated) was demonstrated between week 1 and 2 for many of the variables. This was attributed to changes in sperm reserves and learning which affected many semen variables the 1st week of semen collection.

Results

In this study of rabbits dosed 5 days per week, for 3 weeks, with 100 mg kg^{-1} Vin, there were increasing plasma levels of Vin and M2 throughout the experimental period (Fig. 5). At the conclusion of the study (8 weeks post-dosing) Vin metabolites were still detectable in the plasma, skin and testes of the rabbits. Relatively high concentrations (1200 ng ml^{-1}) of the metabolite M2 were found in the skin of the dosing site (Fig. 6).

The rabbits exposed to Vin had a smaller weight gain during pubertal growth (approaching significance $P=0.059$; Fig. 7). At maturity, the accessory sex glands of the exposed animals weighed less than those of the controls ($P=0.016$; Fig. 8). The smaller accessory sex glands were not merely a reflection of the smaller body weight, as shown in Fig. 9, which depicts the proportional weight of the accessory sex glands to the total body weight. Surprisingly, the pooled sperm count of the exposed animals was significantly higher ($P=0.017$) than that of the unexposed animals (Fig. 10).

Discussion

Some of the most critical factors facing scientists in the evaluation of environmental anti-androgenic effects is the analytical needs of increased speed, repeatability, resolution and automation of sample preparation for analytical detection of chemicals. A method is described that provides a synthesis of new automated technology for sample preparation and analysis utilizing sensitive exposure biomarkers (Table 1).

The significantly lower body weight gain in the treated rabbits indicates general systemic toxicity resulting from the Vin exposure. The mean difference was only 116 g (controls = 2230 g and treated = 2114 g) at termination. The significantly lower weights of the accessory sex glands are probably related to the anti-androgenic effects of Vin. The mean weight for controls was 5.3 g and that for treated animals was 4.3 g ($P=0.016$). The unexpected increase in pooled sperm count found in this study is difficult to interpret. It is possible that the anti-androgenic effects of Vin blocked the negative feedback mechanism of testosterone on the hypothalamus or pituitary gland, resulting in an increase in gonadotrophin release, consequently increasing sperm production at puberty.

This report is the result of a single dose-level study. Review of the blood plasma levels of Vin reveals that the doses produced very low tissue levels (only three of the exposed rabbits demonstrated blood Vin levels above the upper 95% confidence for the controls). However, after multiple doses of Vin, the M2 metabolite, which may be a more potent anti-androgen than the parent compound, Vin, was detectable. More detailed studies that include measurement of endrocrine levels and responses at higher doses are required to properly interpret these findings.

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