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Studies of the Toxicological Potential of Tripeptides (L-Valyl-L-prolyl-L-proline and L-Isoleucyl-L-prolyl-L-proline): IV. Assessment of the Repeated-Dose Toxicological Potential of Synthesized L-Valyl-L-prolyl-L-proline in Male and Female Rats and Dogs

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The objective of these repeated-dose, 8-week studies was to assess the toxicological potential of a synthetic tripeptide, L-valyl-L-prolyl-L-proline (VPP), when administered to Charles River rats and Beagle dogs. Groups of 20 male and 20 female rats were fed powdered diets containing sufficient VPP to afford daily doses of 0, 2, 8, or 16 mg/kg body weight (BW)/day. Groups of five male and five female dogs were administered 0, 2, 8, or 16 mg/kg BW/day in hard gelatin capsules. Antemortem evaluative parameters for both species included grossly observable clinical signs, body weight and food consumption, clinical pathology (hematology, clinical chemistry, urinalysis), and ophthalmological examinations. Dogs also received electrocardiographic examinations. Postmortem evaluations in both species included complete necropsy, determination of major organ weights, and histopathological examination of specimens from approximately 50 organs and tissues. All rats and dogs survived to the scheduled termination of the studies and neither species exhibited evidence of VPP effects on appetite or body weight gain/maintenance. Ophthalmic examinations revealed occasional lens clouding in rats, but this occurred in all groups and was not attributable to VPP. Some clinical pathology parameters in both species were occasionally altered, but there was no evidence that this was dose-related. Electrocardiographic examinations in dogs revealed no VPP-associated changes. Mid- and high-dose male rats (but not females) had slightly reduced mean pituitary and kidney weight parameters, whereas mid- and high-dose females had slightly increased mean uterus:body weight ratios. There were no microscopic correlates for these minor changes. Ten percent to 20% of all female rats (but not males) exhibited corticomedullary mineralization of the kidney and gliosis of the optic nerve, and 10% to 20% of males (but not females) had thymic hemorrhage. Postmortem evaluations of dogs revealed no VPP-related effects on organ weights or either macro- or microscopic appearances of organs. The results of these studies provided no evidence of either local or systemic toxicity. Similarly, there was no evidence of neurotoxicity that might have been detected by the appearance of physical or behavioral changes during gross observations of animals. Although these results do not identify target organs for VPP toxicity, the no-observable-effect level and maximally tolerated dose are both greater than 16 mg/kg/day when administered to male and female rats and dogs for 8 consecutive weeks. Based upon food enhancement levels of VPP currently being evaluated, the resultant margin of safety (160) is substantial.

Keywords Dogs, IPP, Maximum Tolerated Dose, No-Observable-Effect Level, Repeated-Dose Toxicity, Rats, Tripeptide, VPP

INTRODUCTION

The consumption of fermented milk to maintain good health, including the maintenance of normal blood pressure, is an ancient tradition in a number of areas of the world (e.g., East Asia, France). Recent studies have suggested that fermented milk has a normotensive effect in hypertensive rats and humans, but no effect on blood pressure in normotensive humans. However, to date there have been no published results of safety evaluations relevant to a scientifically sound assessment of the safety of

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fermented milk or its biologically active constituents. This paper is one in a series of nine publications that are all presented in the same issue of the *International Journal of Toxicology*. It is the purpose of these publications to bring together the results of the relatively few currently published articles and to publish, for the first time, a significant body of research on the safety/toxicological potential of fermented milk and its active constituents.

Prompted by early scientific results, additional research sought to determine the source of the biological effect and revealed that cardiovascular activity resided in two naturally occurring and well-defined tripeptides, specifically L-valyl-L-prolyl-L-proline (VPP) and L-isoleucyl-L-prolyl-L-proline (IPP). A process for controlling the production of these tripeptides was developed consisting of fermenting reconstituted powdered skim milk using a starter culture containing *Lactobacillus helveticus*.

In 1997, products containing VPP plus IPP were approved for manufacture, sale, and consumption in Japan by the Ministry of Health, Labor, and Welfare, under the Food for Specified Health Use (FOSHU) regulations. These tripeptide-containing products have been sold continuously in Japan since 1997 and include both pasteurized fermented milk drinks and tablets.

The physical and chemical properties of VPP and IPP have been presented in Bernard et al. (2005b). To summarize, VPP (CAS no. 58872-39-2), a white powder with a melting point of 198.5°C, has a molecular weight of 311.4, and a chemical formula of $C_{15}H_{25}N_3O_4$. IPP (CAS no. 26001-32-1) is also a white powder, but with a lower melting point (125.9°C), a molecular weight of 325.3, and a chemical formula of $C_{16}H_{27}N_3O_4$.

Naturally occurring VPP plus IPP is consumed in a number of foods, including dairy products, most notably in cheeses. Estimating its consumption from food sources is difficult owing to variability even within the same food type (e.g., concentrations of VPP plus IPP vary as much as 10-fold in various brands of cheddar cheese). A high, but not unreasonable, estimate of possible VPP plus IPP ingestion from consumption of cheese is 2988 mg/person/year, which is equivalent to 8.2 mg/person/day or 0.11 mg/kg body weight/day (Bernard et al. 2005b). This compares with the average consumption from VPP plus IPP enhanced foods in Japan: 106.8 mg/person/year, which is equivalent to approximately 0.3 mg/person/day or 0.005 mg/kg body weight/day (based upon a 60-kg Japanese individual) (Bernard et al. 2005b).

In a previous publication, scientific data were reviewed and it was concluded that VPP plus IPP was active in the maintenance of normal blood pressure in hypertensive rats and humans, whereas no effect on blood pressure was observed in normotensive rats and humans (Bernard et al. 2005b).

Consideration is being given to enhancing natural VPP plus IPP levels in yogurt for consumption in the United States. This enhancement could result in an increase in mean VPP plus IPP consumption of 118.1 g/person/day or 0.1 mg/kg body weight/day based on a 75-kg average American (Bernard et al. 2005b). Prior to such a consideration, substantial evaluation and study of the safety of VPP plus IPP should be undertaken. It has been reported that the single-oral-dose, no-observable-effect levels for powdered *L. helveticus*–fermented milk, pasteurized casein hydrolysate, and VPP were equal or greater than 4000, 2000, and 400 mg/kg, respectively. Similarly, no signs of toxicity were observed when powdered *L. helveticus*–fermented milk was administered orally for 28 consecutive days at a dose of 2,000 mg/kg (Maeno et al. 2005b). The current publication describes the results of dietary administration of a synthetic tripeptide, VPP, for 8 weeks to both rats and Beagle dogs.

METHODS AND MATERIALS

The Test Article

The test article for these experiments was a synthesized tripeptide, L-valyl-L-prolyl-L-proline (VPP). VPP was mixed with powdered Rat and Mouse No. 1 Maintenance Diet RM1 (Special Diets Services Ltd., Essex, England) and administered to rats in concentrations estimated to deliver daily doses of 0, 2, 8, or 16 mg/kg of the tripeptide. Diet mixes were freshly prepared each week and adjustments in concentrations of VPP were based on the most recently determined mean body weights of animals.

A validated liquid chromatography–mass spectrometry (LC-MS)/MS method was used to analyze diet mixes to verify both homogeneity and concentrations of VPP. Analyses revealed that homogeneity in three layers of the diet mix (top, mid, and bottom) was consistently within 7.5% of the nominal concentrations (data not shown). The results of verification of VPP concentrations in the diets mixed during experimental weeks 1 and 7 are shown in Table 1. Concentrations of VPP ranged from 89% to 107% of target indicating excellent precision in diet preparation.

VPP was administered to dogs in hard gelatin capsules that had been prepared weekly. Capsules were individualized for each dog and the amount in each capsule was calculated to deliver daily doses of 0, 2, 8, or 16 mg/kg of VPP, based on the most recently recorded individual animal body weight. These doses were chosen based upon the following. The estimated daily dosage for the VPP as a dietary supplement could be as high as 5 to 10 mg/day. For a 60-kg adult, this represents an intake of approximately 0.09 to 0.17 mg/kg/day. The highest dosage tested in the 8-week rat and dog repeated-dose studies was 16 mg/kg/day and represents approximately 100 to 200 times the maximum estimated daily consumer intake.

LC-MS/MS Analysis

Chromatographic analyses employed Micromass Quattro LC controlled directed by a Mass Lynx data system. A Columbus 5 micro C18 column (150 mm × 2 mm internal diameter) was employed for the quantitative analysis of VPP. The solvent system employed in the analysis consisted of (a) 1% formic acid in water and (b) 1% formic acid in water/acetonitrile 10:90 (v/v).

TABLE 1
Verified concentrations of VPP in rodent diet fed to male and female CD rats for 8 consecutive weeks

Sex for which the diet mix was intended	Target concentration (ppm)[a]	Mean concentration (ppm)[b]	Percent of target
Experimental week 1			
Males	0	Not detected	—
	14	13.1	93.6
	56	53.7	95.9
	112	121	108
Females			
	0	Not detected	—
	16	14.2	88.8
	64	59.1	92.3
	127	136	107
Experimental week 7			
Males	0	Not detected	—
	28	27.4	97.9
	110	117	106
	221	226	102
Females	0	Not detected	—
	22	23.6	107
	89	91.4	103
	180	187	104

[a] Target concentrations of VPP were adjusted weekly to maintain test agent consumption at the test doses (0, 2, 8, and 16 mg/kg/day).

[b] Actual concentrations were determined during assessments of homogeneity of mixed diets. Homogeneity was assessed by determining the concentrations of VPP in three layers of the diet mix with a validated LC-MS/MS assay. Actual concentrations of the diet mixes were determined as the mean of the results of the nine assays per diet.

VPP was analyzed using an elution gradient, 100% of A ($t = 0$ min), 20% of A ($t = 6$ min), 20% of A ($t = 12$ min), and 100% of A ($t = 13$ min). VPP was quantified using the L-isoleucyl-L-prolyl-L-proline (IPP) peptide as the internal standard. VPP was extracted from a 5 g sample of diet mix by shaking with 1% formic acid solution (100 ml) for 30 min. The diet extract was further diluted to result in a concentration within the range 40 to 80 ng/ml. IPP, added to the filtrated diet extract (50 ng/ml), served as the internal standard. Twenty microliters were applied onto the column and analyzed at 55°C using a flow rate of 0.2 ml/min. A mass spectrometer (The Micromass quattoro LC) was operated in positive ion MS/MS mode for analysis of VPP and IPP using the transition m/z 312.1 → 197.1 and 326.1 → 213.1, respectively.

Eight-Week Study in Rats

Male and female CD rats, 25 to 29 days of age, were purchased (Charles River, UK) and acclimated to laboratory conditions for 15 days. They were individually housed in suspended plastic cages with stainless steel mesh tops and bottoms. Room conditions were 19°C to 25°C, 40% to 70% relative humidity, with at least 15 air changes per hour and a 12-h on:12-h off light cycle. Animals had free access to food except when urine was being collected and before blood sampling for clinical pathology assessments. Municipal water from polycarbonate bottles with sipper tubes was available at all times except when urine was being collected.

Animals were randomly assigned to one of four groups, each containing 20 males and 20 females, and were administered VPP (0, 2, 8, or 16 mg/kg/day) in powdered diet. Control and mixed diets were available 7 days per week for 8 consecutive weeks.

Animals were observed for clinical signs at least twice daily. Detailed observations were performed weekly and included assessments of behavior and thorough examination of physical condition. Each animal was weighed once weekly during the period of acclimation (on day 1 of dosing) and weekly thereafter. Weekly food consumption was estimated by weighing food remaining in the feed hopper at the end of the week. Efficiency of food utilization (EFU; grams of body weight gained per 100 g of feed consumed) was computed weekly.

During treatment week 7, urine was collected from the rats by placing them in individual metabolism cages from 1530 h until 0830 h the following day, during which time food and water was withheld. During the final week of treatment, each animal was subjected to ophthalmic examination. Eyes were treated with Mydracil, and examined with an indirect ophthalmoscope. Ophthalmic structures examined included palpebrae and adjacent structures, conjunctiva, cornea and sclera, anterior chamber and iris, lens and vitreous, and the ocular fundus.

Also during the final week, each animal was anesthetized with isoflurane/nitrous oxide and blood samples for hematologic and clinical chemistry assessments were collected via retro orbital sinus puncture after overnight fasting. Clinical pathology parameters are summarized in Table 2. In addition, prothrombin times were determined and blood smears were prepared with Romanowsky stain and examined for abnormal cell types.

After the final treatment day, animals were sacrificed with carbon dioxide and subjected to thorough necropsies with approximately 50 organs and tissues being collected. Major organs were weighed and collected tissues were preserved in 10% neutral buffered formalin. The disposition of tissues and organs is summarized in Table 3.

For each variable and time point, a preliminary assessment of normality and homogeneity of variance was made using the R1-squared test (Selwyn and Gaccione 1993). If the test was not significant at the 5% level, then groups were compared using William's test (1971, 1972). If the test was significant at the 5% level, then groups were compared using Shirley's nonparametric trend test (1977). Where 75% or more of the values for a given variable were the same, an exact permutation trend test was used to assess significance. Intergroup differences in histopathology were assessed using a Cochran-Armitage test (Armitage 1955).

TABLE 2
Clinical pathology parameters assessed in male and female rats and dogs administered VPP for 56 consecutive days

	Hematologic parameters	
Hematocrit	Hemoglobin concentration	RBC count
Total and differential WBC count	Platelet count	Mean cell hemoglobin concentration (MCHC)
Mean cell hemoglobin (MCH)	Mean cell volume (MCV)	Percent reticulocytes (dogs only)
Prothrombin time (dogs only)	Activated partial thromboplastin time (dogs only)	
	Clinical chemistry parameters	
Alkaline phosphatase	Alanine aminotransferase	Aspartate aminotransferase
Gamma-glutamyl transpeptidase (rats only)	Ornithine carbamyl transferase (rats only)	Glucose
		Total triglycerides
Total bilirubin	Total cholesterol	Total protein
Creatinine	Urea	Sodium
Albumin	Globulin	Chloride (dogs only)
Calcium	Inorganic phosphate	
Potassium (dogs only)		
	Urinalysis	
Volume	pH	Specific gravity
Protein	Glucose	Sodium
Microscopic sediment	Potassium (dogs only)	Chloride (dogs only)
Ketones (dogs only)	Bilirubin (dogs only)	Blood pigments (dogs only)

Eight-Week Study in Dogs

Twenty male and 20 female beagle dogs (Harlan France, F-03800 Gannat, France) were acclimated to laboratory conditions for 5 weeks. Their age at the start of treatment was 23 to 25 weeks, and their body weights ranged from 7.2 to 9.7 kg for males and 5.6 to 8.3 kg for females. During acclimatization, the animals were inoculated against canine distemper virus, canine hepatitis virus, canine parvovirus, *Leptospira canicola, Leptospira icterohaemorrhagiae*, and *Bordetella bronchiseptica*.

Dogs were housed in kennels (5- to 6-m^2 floor area) at 15°C to 24°C, with a 12-h on:12-h off light cycle. Each animal was offered 400 g of dry diet (Special Diet Services, Ltd.) daily and water (via an automatic water valve) was available ad libitum except during the collection of urine (twice during the pretreatment period and once during treatment week 8). During dosing period, food was offered approximately 15 to 20 min after dosing. The animal had access to food for about 3 h each day. Any uneaten food was removed from the kennels at approximately 1300 h and any food remaining was recorded and discarded.

The dogs were randomized into four groups, each containing five dogs of each sex and administered daily oral doses of VPP (0, 2, 8, or 16 mg/kg in hard gelatin capsule) 7 days a week for 8 consecutive weeks.

Evaluative parameters during the antemortem portion of the study included determination of weekly bodyweights and food consumption; gross observation of clinical signs before and after dosing; and weekly physical examinations. Each animal received electrocardiographic and ophthalmoscopic examinations during the pretreatment period and during experimental week 8. Electrocardiographic parameters included wave intervals (P, PR, QRS, ST, QT, and QTc) and amplitudes (R, Q, R, S, and T). Ophthalmic examinations included all visible portions of the eye: adnexa, conjunctiva, cornea and sclera, anterior chamber, and iris (pupil dilated), lens, vitreous and ocular fundus.

Clinical laboratory examinations (hematology, blood chemistry, and urinalysis) were conducted during the pretreatment period and the final week of dosing. Food was removed overnight before collection of blood samples and during the period of urine collection. Samples of venous blood were drawn from the jugular or cephalic vein from all animals in treatment and control groups. Blood samples were collected over EDTA (hematological investigations), citrate (coagulation tests), or heparin (blood chemistry investigations). Urine samples were collected in containers over a period of approximately 16 h from all animals, water having been withheld from the animals about 5 h prior to the start of collection. Clinical pathology parameters assessed are listed in Table 2.

Upon completion of 8 weeks of dosing with VPP, animals were fasted overnight and sacrificed with an overdose of pentobarbital. Each animal was subjected to a thorough necropsy in which superficial tissues were examined both visually and by palpation; the cranial roof was removed to allow observation of the brain, pituitary gland, and cranial nerves. Abdominal viscera were examined before and after removal and the urinary bladder was examined externally and internally. Major organs were weighed and approximately

TABLE 3
Disposition of tissues and organs from male and female rats and dogs dosed for 56 consecutive days with VPP

	Organs weighed	
Adrenal glands	Brain	Epididymides
Heart	Kidneys	Liver
Ovaries	Pituitary	Prostate
Salivary glands (submaxillary)	Seminal vesicles (rats only)	Spleen
Testes	Thymus	Thyroid and parathyroids
Uterus with cervix	Lung (dogs only)	
	Organs collected and preserved[a]	
Adrenals	Aorta (thoracic)	Brain
Cecum	Colon	Duodenum
Epididymides	Eyes (with optic nerve, dogs only)	Fallopian tubes (rats only)
Femur	Harderian gland (rats only)	Heart
Ileum	Jejunum	Kidneys
Lachrymal glands (rats only)	Larynx (rats only)	Liver
Lung with mainstream bronchi	Lymph nodes (mandibular, mesenteric)	Mammary gland (caudal)
Oesophagus	Optic nerves	Ovaries
Pancreas	Peyer's patches (rats only)	Pharynx (rats only)
Pituitary	Prostate	Rectum (rats only)
Salivary glands (submaxillary)	Sciatic nerve	Seminal vesicles
Skeletal muscle (thigh)	Skin	Spinal cord
Spleen	Sternum	Stomach
Testes	Thymus	Thyroid and parathyroids
Tongue	Trachea	Urinary bladder
Uterus with cervix	Vagina	Zymbal's gland (rats only)
Gall bladder (dogs only)	Nictitans glands (dogs only)	ID tattoo (dogs only)
	Tissues prepared for microscopic examination[b]	
Adrenals	Aorta (dogs only)	Brain
Femur	Gall bladder (dogs only)	Heart
Liver	Lungs	Kidneys
Mammary glands	Nictitans gland (dogs only)	Spinal cord
Sternum	Stomach	Thyroid
Uterus		

[a]Samples of all collected organs and tissue abnormalities were preserved in neutral buffered formalin, except for eyes, which were preserved in Davidson's fixative and testes and epididymides in Bouin's fluid.

[b]Tissues examined microscopically were dehydrated, embedded in paraffin, sectioned, and processed into slides then stained with H&E.

50 organs and tissues were collected and preserved. The disposition of organ samples collected during necropsy is shown in Table 3.

For each variable and time-point, a preliminary assessment of normality and homogeneity of variance over sex-by-dose group combinations was made using the R1-squared test (Selwyn and Gaccione 1993; Blom 1958). If the R1-squared test was not significant at the 5% level, then a two-way analysis of variance with sex, dose, and sex-by-dose interaction was performed. If the sex-by-dose interaction term was not significant at the 1% level, then the two-way analysis was retained. If the interaction term was significant, then only a separate-sex analysis was performed. Groups were compared using William's test (1971, 1972) in both cases. If the R1-squared test was significant, then, for separate sexes only, the groups were compared using Shirley's nonparametric trend test (1977). Where 75% or more of the values for a given variable were the same, an exact permutation trend test was used to assess significance.

RESULTS

Eight-Week Study in Rats

Test Article Consumption

Food intake and the results of the diet analyses were used to estimate the average daily doses of VPP (Table 4). The results

TABLE 4
Average daily doses of VPP administered to male and female CD rats for 8 consecutive weeks

	Target daily dose (mg/kg) of VPP			
	0	2.0	8.0	16
Actual dose to males	0	2.0 ± 0.1	7.8 ± 0.4	15.8 ± 0.8
Actual dose to females	0	2.0 ± 0.1	8.1 ± 0.4	15.9 ± 1.0

of this analysis revealed that actual doses of VPP administered to male and female rats ranged from 98% to 101% of target.

Clinical Toxicology Parameters

All rats in this experiment survived until the scheduled termination of the study. Gross observations of animals revealed sporadic incidences of hair loss, staining of fur, and perigenital staining. These signs appeared in both control and dosed animals and there was no appearance of dose-response relationships. These minor signs were considered to be spontaneous and of no interpretative utility.

Body weight gain data are summarized in Figure 1. All animals gained weight during the study and there were no statistically significant differences between dosed and control rats of either sex. Consistent with the body weight gain data, neither weekly food consumption nor EFU were affected by the presence of VPP in the diet. Food consumption data are not shown, but group mean EFU values are shown in Figure 2. The mean EFU values for mid- and high-dose female rats during week 6 were significantly lower (32%, $p < .05$) than that of the control group, but mean EFU values for the overall study were unaffected by administration of VPP.

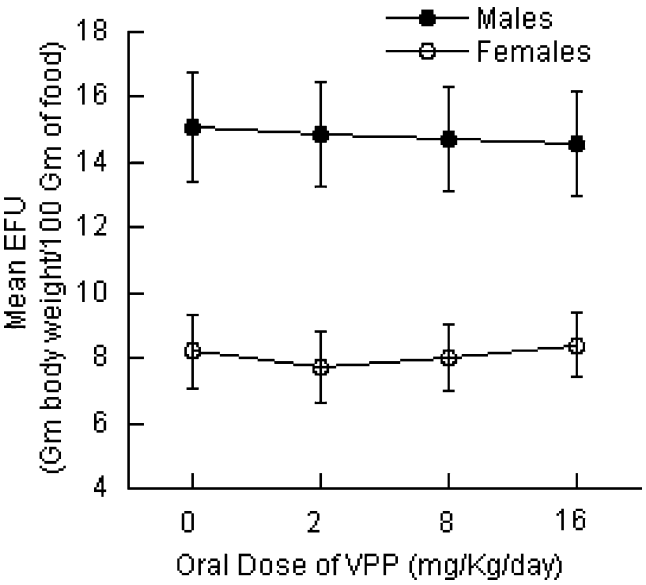

FIGURE 2
Mean efficiency of food utilization (EFU) for groups of 20 male and 20 female rats administered VPP (0, 2, 8, or 16 mg/kg/day) in their diets. Statistical analysis of the data (ANOVA) revealed that overall EFU during the 8-week experiment was unaffected by VPP administration.

Ophthalmic examinations revealed occasional abnormalities in all groups of animals and none were considered attributable to administration of VPP. Corneal and lenticular opacities were observed in some animals both before and after treatment, although they were observed more frequently during week 8. The incidences of abnormalities were evenly distributed among dosed and control groups. The results of urinalysis also revealed neither statistically significant nor biologically meaningful differences between dosed and control animals.

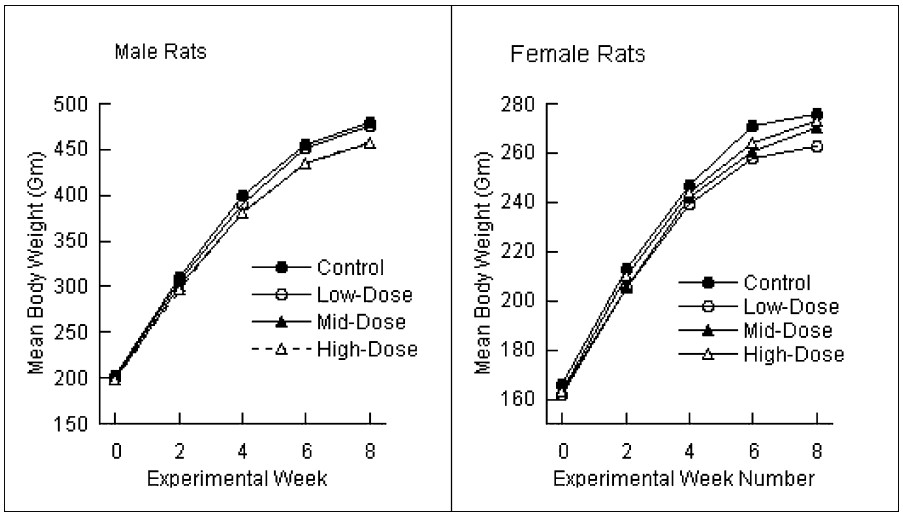

FIGURE 1
Mean body weight gains by male and female rats administered VPP (0, 2, 8, or 16 mg/kg/day) for 8 consecutive weeks. Each data point represents the mean body weight of groups of 20 animals. Statistical analysis of the data (ANOVA) revealed no significant differences between groups.

TABLE 5
Statistically significant changes in hematologic parameters in male and female CD rats dosed for 8 consecutive weeks with VPP

Experimental group (mg/kg/day)	Large unstained cells (10^9/L)		Monocytes (10^9/L)		MCHC (g/dl)	
	Males	Females	Males	Females	Males	Females
Controls	0.27 ± 0.12	0.13 ± 0.05	0.35 ± 0.10	0.24 ± 0.10	34.6 ± 0.5	34.7 ± 0.5
2	0.24 ± 0.10	0.11 ± 0.04	0.35 ± 0.13	0.22 ± 0.09	34.8 ± 0.3	34.4 ± 0.5
8	0.23 ± 0.08	0.08 ± 0.04*	0.36 ± 0.10	0.11 ± 0.05*	34.8 ± 0.4	34.5 ± 0.4
16	0.20 ± 0.07*	0.12 ± 0.04*	0.30 ± 0.09	0.22 ± 0.09*	34.6 ± 0.4	34.3 ± 0.4**

Significantly different from controls: * $p < .05$; ** $p < .01$.

Hematology Assessment

Hematologic changes observed in VPP-dosed rats are summarized in Table 5. High-dose males and mid- and high-dose females had significantly reduced mean numbers of large unstained cells. The reductions ranged from 26% in males to as high as 39% in mid-dose females, but only 1% in high-dose females. The absence of evidence of a dose dependency for these changes indicates that they are most likely not VPP-related. Mid- and high-dose females also exhibited significantly reduced mean monocyte counts (54% and 8%, respectively), but the mean monocyte counts in groups of dosed males were not different from that in the control group. Mean cell hemoglobin concentration was slightly (approximately 1%), but significantly ($p < .05$), reduced in all groups of dosed females; however, there was no similar decrease in males.

Clinical Chemistry Assessments

Changes in clinical chemistry parameters in VPP-dosed rats were sporadic, of small magnitude and of doubtful toxicological significance (Table 6). High-dose males had an increase in serum γ-glutamyltransferase (γ-GT) and a 29% decrease in ornithine carbamyl transferase, but these parameters were unaffected in females. Total bilirubin was reduced by 33% in all dosed female groups, whereas this parameter was unaffected in males. Small (1%) increases in serum sodium concentrations in all dosed females were statistically significant ($p < .05$), but there was no similar change in dosed males. Mean serum calcium concentration was increased (3%) in high-dose females and significantly decreased (2% to 3%) in mid- and high-dose males. The high-dose was associated with a significant ($p < .05$) 6% increase in serum glucose concentration in both males and females; however, there was no evidence of a dose-response relationship.

Postmortem Parameters

Organs removed and weighed are listed in Table 3. Using body weights and brain weight, relative organ weights were calculated. Organ weight parameters for pituitary and kidney (males only) and uterus were significantly different in groups of VPP-dosed animals (Table 7). Mean absolute and relative (to brain) male pituitary weights were decreased in mid- and high-dose animals whereas mean relative (to brain) kidney weights were also decreased in mid- and high-dose males. The only statistically significant changes in females were increased mean uterus weights relative to body weight in mid- and high-dose groups. Other absolute weights, organ to body and organ to brain ratios, were unaffected by the test article (data not shown).

Macroscopic examination of animals during necropsy revealed a small number of spontaneous changes commonly observed in rats. Changes included liver masses in 1/20 control males and 1/20 high-dose females, darkened areas on the thymus gland of 2 to 5 animals in all but the low- and mid-dose female groups, and fluid distension of the uterus of 3 to 11 females of each group. None of these changes was attributable to administration of VPP.

Microscopic examination of approximately 50 organs and tissues from dosed and control rats revealed only changes considered to be spontaneous in nature (Table 8). Ten percent to 20% of control and dosed females exhibited corticomedullary mineralization of the kidney and gliosis of the optic nerve, but neither of these changes was observed in males. Fifteen percent to 55% of females in all dose groups also exhibited uterine dilation. Male rats in all groups (10% to 20%) had thymic hemorrhage, but, among females, only 3/20 in the high-dose group exhibited thymic hemorrhage. Chronic inflammation of the Harderian gland was observed in all groups of males (15% to 30%) and in all but the low-dose group of females (5% to 15%).

Eight-Week Study in Dogs

Clinical Toxicology Parameters

All dogs survived until the scheduled termination of the study. A variety of clinical signs which included loose stool, lacrimation, dermal rashes, abrasions and scabs, and occasional emesis appeared equally in control and dosed animals. There were no clinical signs attributable to administration of VPP. Individual animals all gained weight during the 8-week dosing period and VPP was without effect

TABLE 6

Statistically significant changes in clinical blood chemistry parameters in male and female CD rats dosed for 8 consecutive weeks with VPP[a]

Experimental group (mg/kg/day)	γ-Glutamyl transpeptidase (U/L)		Ornithine carbamyl transferase (U/L)		Total bilirubin (μmol/L)		Glucose (mmol/L)		Potassium (mmol/L)		Sodium (mmol/L)		Calcium (mmol/L)	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Control	0 ± 1.1	0 ± 0.4	3.5 ± 1.5	4.4 ± 3.1	2 ± 0.6	3 ± 0.6	7.0 ± 1.0	6.8 ± 0.8	4.0 ± 0.2	3.9 ± 0.3	142 ± 2	139 ± 1	2.85 ± 0.08	2.74 ± 0.09
2	1.0 ± 2.0	0 ± 0.4	3.2 ± 1.3	4.8 ± 3.5	3 ± 0.7	$2 \pm 0.6^{**}$	6.6 ± 0.7	6.8 ± 0.7	4.1 ± 0.3	3.7 ± 0.3	142 ± 2	$140 \pm 2^{*}$	2.85 ± 0.09	2.73 ± 0.10
8	0 ± 0	1.0 ± 1.2	3.0 ± 2.2	6.8 ± 7.9	3 ± 0.8	$2 \pm 0.5^{**}$	6.8 ± 0.6	7.0 ± 0.8	4.1 ± 0.2	3.8 ± 0.2	142 ± 2	$141 \pm 2^{**}$	$2.78 \pm 0.08^{*}$	2.65 ± 0.08
16	$2.0 \pm 1.7^{**}$	0 ± 0.4	$2.5 \pm 1.6^{*}$	6.8 ± 6.4	3 ± 0.7	$2 \pm 0.5^{**}$	7.4 ± 1.4	$7.2 \pm 0.3^{*}$	$4.3 \pm 0.3^{**}$	$4.1 \pm 0.3^{**}$	141 ± 2	$140 \pm 1^{**}$	$2.81 \pm 0.08^{*}$	2.80 ± 0.08

[a]Data expressed as mean $\pm$ *SD*.

Significantly different from control: $^{*}p < .05$; $^{**}p < .01$.

TABLE 7
Statistically significant changes in organ weight parameters in male and female CD rats dosed for 8 weeks with VPP[a,b]

Organ weight parameter	Doses administered to male rats (mg/kg/day)				Doses administered to female rats (mg/kg/day)			
	Control	2	8	16	Control	2	8	16
Pituitary weight (mg)	12.0 (3.0)	11.6 (2.3)	10.5 (2.0)	10.4 (2.0)*	12.5 (1.8)	11.9 (2.8)	12.7 (2.8)	12.5 (2.8)
Pituitary % of brain weight	5.7 (1.4)	5.5 (1.1)	5.1 (1.1)	5.0 (0.9)*	6.5 (1.0)	6.3 (1.5)	6.7 (1.5)	6.4 (1.4)
Uterus % of body weight	—	—	—	—	0.21 (0.07)	0.26 (0.12)	0.33 (0.12)*	0.25 (0.11)*
Kidney% of brain weight	1.6 (0.13)	1.5 (0.13)	1.5 (0.12)*	1.5 (0.10)*	1.1 (0.08)	1.0 (0.15)	1.1 (0.10)	1.1 (0.09)

[a]Only statistically significant data are shown.
[b]Data expressed as mean ± *SD*.
*Significantly different from controls, $p < .05$.

TABLE 8
Microscopic changes observed in male and female CD rats dosed for 8 consecutive weeks with VPP[a]

Organ/observed changes	Dose of VPP administered to male rats (mg/kg/day)				Dose of VPP administered to female rats (mg/kg/day)			
	0	2	8	16	0	2	8	16
Kidney: corticomedullary mineralization	0/20	0/20	0/20	0/20	3/20 (15%)	2/20 (10%)	4/20 (20%)	4/20 (20%)
Optic nerve: gliosis	0/19	0/20	0/20	0/19	4/20 (20%)	3/20 (15%)	2/20 (10%)	2/20 (10%)
Uterus: dilated	—	—	—	—	3/20 (15%)	6/19 (32%)	11/20 (55%)	6/20 (30%)
Thymus: hemorrhage	3/20 (15%)	4/20 (20%)	2/19 (11%)	2/20 (10%)	0/20	0/20	0/20	3/20 (15%)
Harderian gland: chronic inflammation	3/20 (15%)	4/20 (20%)	4/20 (20%)	6/20 (30%)	3/20 (15%)	0/20 (0%)	1/20 (5%)	2/20 (10%)

[a]Data expressed as incidence of change/number of animals examined microscopically.

on magnitudes of weight gained (Figure 3). Food consumption within sexes was identical in control and dosed groups (Figure 4).

Analysis of electrocardiographic recordings, obtained prior to treatment and 2 and 24 h after administration of VPP during week 7, revealed no treatment-associated changes in either wave intervals or amplitudes (data not shown).

The only statistically significant change detected during urinalysis was an increase in mean specific gravity in all groups of dosed males during week 8 (Table 9). Mean specific gravities of dosed females were numerically higher, but statistically identical, to those of controls.

Clinical Pathology Assessments

The results of all clinical pathology assessments in dogs (hematology and serum chemistry) were analyzed by sex. Tabulated clinical pathology results are presented in Tables 10 and 11. Both pretreatment (week 1) and treatment week 7 data are shown for purposes of comparison. Data for both sexes are presented although only one of the sexes may have exhibited statistically significant changes.

Hematology Assessments

Statistically significant changes in the hematologic parameters of VPP-dosed animals are summarized in Table 10. No

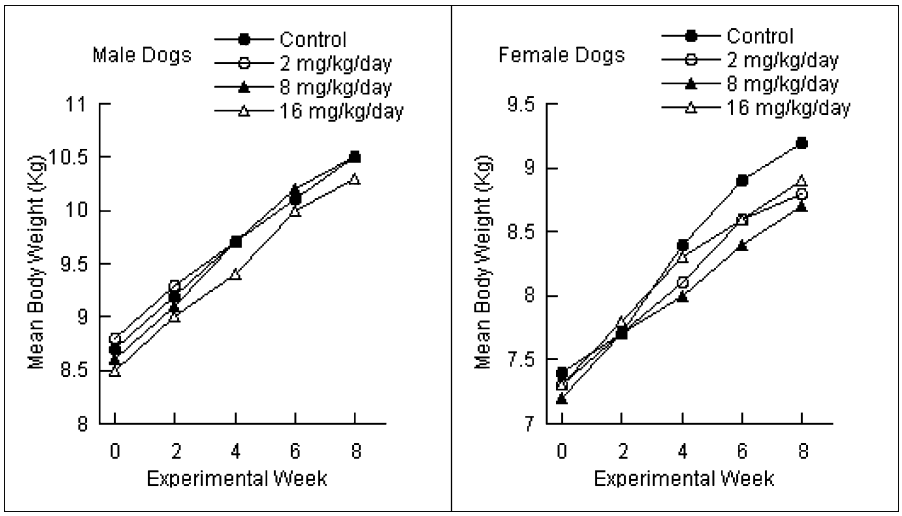

FIGURE 3
Mean body weight gains by male and female beagle dogs administered VPP (0, 2, 8, or 16 mg/kg/day) for 8 consecutive weeks. Each data point represents the mean body weight of groups of five animals. Statistical analysis of the data (ANOVA) revealed no significant differences between groups.

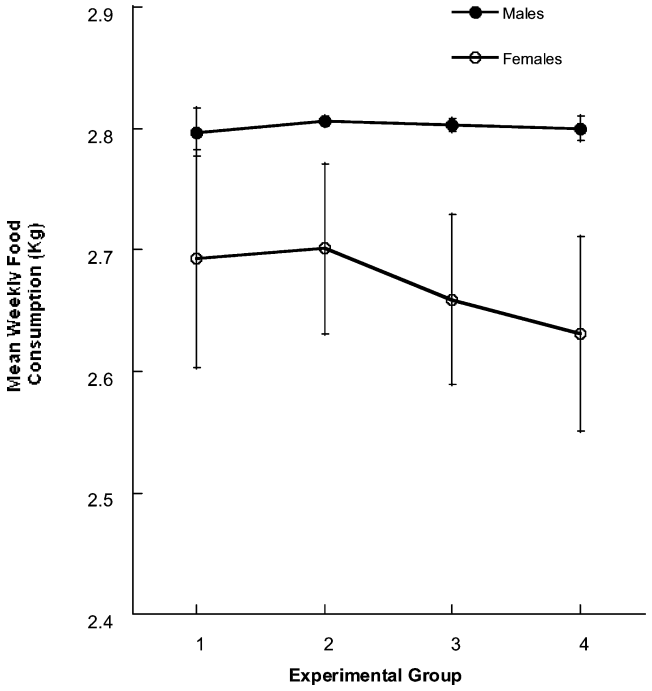

FIGURE 4

Mean weekly food consumption by groups of five male and five female beagle dogs administered oral doses of 0, 2, 8, or 16 mg/kg/day (experimental groups 1, 2, 3, and 4, respectively) of VPP by oral capsule. Statistical analysis of the data (ANOVA) revealed no significant differences in food consumption between dosed groups of the same sex.

significant differences were observed in the hematologic parameters of male dogs. During treatment week 7, VPP-dosed females had statistically significant ($p < .05$) reductions (31% to 40%) in mean neutrophil counts.

TABLE 9

Mean specific gravities of urine from male and female beagle dogs dosed for 8 weeks with VPP[a]

Dose of VPP (mg/kg/day)	Specific gravity pretreatment	Specific gravity week 8
	Males	
0	1041 ± 2.1	1042 ± 1.5
2	1045 ± 7.0	1046 ± 3.1
8	1041 ± 5.4	1045 ± 2.5
16	1041 ± 0.9	1049 ± 7.5*
	Females	
0	1041 ± 3.4	1043 ± 1.3
2	1041 ± 2.9	1049 ± 7.8
8	1040 ± 3.2	1044 ± 4.6
16	1040 ± 2.8	1045 ± 2.7

[a]Data expressed as g/L ± *SD*.

*Significantly greater than concurrent control ($p < .05$).

Clinical Chemistry Assessment

No statistically significant deviations in clinical chemistry parameters were apparent in either sex (Table 11).

Postmortem Parameters

Like clinical pathology data, organ weight data for dogs were analyzed by sex, then with sexes combined. When statistically significant differences in organ weight parameters were detected, all three data sets were presented in Table 12.

Organ weight parameters in dogs were generally unaffected by administration of VPP. Statistically significant ($p < .05$) increases (35% to 45%) were detected in all female, high-dose group ovary weight parameters (absolute and relative weights). The only other statistically significant variations were observed during analysis of the sexes' combined thyroid weight parameters. The high dose was associated with significant ($p < .05$) decreases (20% to 30%) in absolute and relative thyroid weights.

Macroscopic examination of each dog yielded a small number of deviations in organ coloration, small hemorrhagic areas, cysts, and evidence of organ congestion. These changes were distributed throughout all groups without evidence of a relationship to administration VPP.

Microscopic examinations revealed no evidence of VPP-induced changes. Key findings are summarized in Table 13. Dosed males had a higher incidence of heart valve hemocysts, but this change was also observed in a control male. There was no evidence of a dose-response relationship for this change and it is considered to be without toxicological significance. A high incidence (80% to 100%) of renal papillary mineralization was observed in both sexes of control and dosed dogs, and cytoplasmic vacuolation of the proximal convoluted tubules was also evenly distributed among dosed and control dogs. The severity of these renal changes was judged to be minimal in all cases.

All females in all experimental groups were considered to have immature ovaries. This is most likely related to the relatively young age of the animals at the initiation of the study (approximately 25 weeks).

Minimal to slight thymic involution/atrophy was observed in at least one dog in all but the mid-dose female group. Although the numerical incidence of the change appeared to be somewhat higher in mid- and high-dose males (3/5, 60% in each group), the severity of the change was generally less than that observed in the 1/5 (20%) male control dog.

DISCUSSION

Eight-Week Study in Rats

Dietary administration of VPP in powdered diets for 8 weeks at a top dose of 16 mg/kg/day resulted in no evidence of physical, biochemical, or histopathological changes associated with the administration of the test article. Statistically significant variations in hematologic and biochemical parameters were small,

TABLE 10
Statistically significant hematologic changes observed in male and female beagle dogs dosed with VPP for 8 weeks

	Pretreatment values (week 1)				Values during week 7			
Dose of VPP (mg/kg/day)	% Reticulocytes	Neutrophils (10^9/L)	WBC (10^9/L)	Eosinophils (10^9/L)	% Reticulocytes	Neutrophils (10^9/L)	WBC (10^9/L)	Eosinophils (10^9/L)
				Males				
0	0.88 ± 0.27	8.3 ± 2.5	14.8 ± 2.9	0.47 ± 0.19	0.83 ± 0.09	7.2 ± 1.8	12.5 ± 1.8	0.42 ± 0.11
2	0.69 ± 0.21	8.5 ± 2.1	15.1 ± 2.6	0.39 ± 0.16	0.68 ± 0.27	7.0 ± 1.4	12.5 ± 1.9	0.35 ± 0.16
8	0.74 ± 0.21	6.9 ± 1.0	12.8 ± 2.0	0.32 ± 0.20	0.75 ± 0.20	7.2 ± 1.0	12.7 ± 0.3	0.31 ± 0.04
16	0.62 ± 0.13	7.7 ± 1.5	14.4 ± 2.3	0.26 ± 0.07	0.71 ± 0.17	7.1 ± 1.1	12.9 ± 2.1	0.19 ± 0.09
				Females				
0	0.87 ± 0.29	8.1 ± 0.8	14.4 ± 1.6	0.30 ± 0.24	1.06 ± 0.14	11.0 ± 2.5	17.6 ± 3.8	0.35 ± 0.27
2	0.95 ± 0.18	6.6 ± 0.8	11.7 ± 1.8	0.24 ± 0.10	0.74 ± 0.15	7.0 ± 2.0*	11.9 ± 2.8	0.26 ± 0.04
8	0.58 ± 0.39	7.6 ± 1.4	14.3 ± 3.9	0.23 ± 0.05	0.84 ± 0.26	6.7 ± 0.5*	12.2 ± 1.6	0.24 ± 0.06
16	0.50 ± 0.18	6.8 ± 1.4	12.6 ± 1.5	0.42 ± 0.26	0.73 ± 0.21	7.6 ± 1.0*	13.0 ± 1.5	0.31 ± 0.13

*Significantly different from controls ($p < .05$).

lacked relationships to doses, and were generally observed in only one sex.

Mean absolute and relative (to brain) pituitary gland weights were significantly decreased in mid- and high-dose male rats. However, the magnitudes of decreases were the same in both dosed groups (i.e., not consistent with dose-response) and there were neither similar changes in females nor corroborating microscopic changes. These minor pituitary weight changes are not considered treatment-related, but are attributed to normal background random variability.

A small decrease in mean relative (to brain) kidney weights in mid- and high-dose group males was observed in the absence of significant changes absolute weights of either the kidneys or brains. These findings were not observed in females nor were microscopic renal changes observed in either males or females. Based upon this combination of findings, the authors concluded that these changes are not likely to be toxicologically meaningful.

A similar situation was noted with regard to elevations in mean relative uterine weights of mid- and high-dose female rats in the absence of an effect on mean absolute uterine weights. Microscopically, the incidence of dilated uterus was two to three times greater in the mid- and high-dose groups than in the controls and, whereas this phenomenon might offer an explanation for the relative uterine weight difference, it seems unlikely that the change can be attributed to administration of VPP.

TABLE 11
Statistically significant clinical chemistry changes observed in male and female beagle dogs dosed with VPP for 8 weeks

	Pretreatment values (week 1)		Values during week 7	
Dose of VPP (mg/kg/day)	Sodium (mmol/L)	Chloride (mmol/L)	Sodium (mmol/L)	Chloride (mmol/L)
		Male dogs		
0	146 ± 2.8	114 ± 2.2	145 ± 1.1	116 ± 1.5
2	146 ± 0.9	117 ± 1.6	145 ± 1.3	116 ± 0.8
8	144 ± 2.4	113 ± 1.6	145 ± 0.8	115 ± 1.7
16	146 ± 1.1	114 ± 2.3	146 ± 1.4	116 ± 1.3
		Female dogs		
0	146 ± 0.5	117 ± 1.3	144 ± 1.2	114 ± 1.3
2	147 ± 0.9	116 ± 0.5	145 ± 0.9	116 ± 2.3
8	146 ± 1.9	115 ± 1.1	145 ± 2.9	116 ± 2.3
16	147 ± 2.2	116 ± 0.8	147 ± 1.8	117 ± 0.5

Significantly different than combined control: * $p < .05$; ** $p < .01$.

TABLE 12

Statistically significant organ weight parameter changes observed in male and female beagle dogs dosed with VPP for 8 weeks[a]

	Doses administered to male dogs (mg/kg/day)				Doses administered to female dogs (mg/kg/day)			
Organ weight parameter	Control	2	8	16	Control	2	8	16
Ovaries								
Absolute weight (g)	—	—	—	—	0.72 (0.17)	0.92 (0.15)	0.81 (0.07)	0.97 (0.23)*
% of body weight	—	—	—	—	0.008 (0.002)	0.011 (0.001)	0.009 (0.000)	0.011 (0.003)*
% of brain weight	—	—	—	—	0.9 (0.2)	1.2 (0.2)	1.1 (0.1)	1.3 (0.3)*
Thyroid								
Absolute weight (g)	1.1 (0.13)	0.9 (0.11)	1.0 (0.18)	0.8 (0.08)	0.9 (0.09)	0.8 (0.23)	1.0 (0.17)	0.8 (0.21)
% of body weight	0.01 (0.001)	0.008 (0.001)	0.01 (0.002)	0.008 (0.000)	0.01 (0.002)	0.009 (0.003)	0.01 (0.002)	0.009 (0.002)
% of brain weight	1.2 (0.2)	1.1 (0.1)	1.2 (0.2)	1.0 (0.1)	1.2 (0.2)	1.0 (0.3)	1.3 (0.3)	1.0 (0.3)

[a]Data expressed as group mean (*SD*).
*Significantly different from controls: $^{*}p < .05$; $^{**}p < .01$.

TABLE 13

Microscopic changes observed in male and female dogs dosed for 8 consecutive weeks with VPP[a]

	Dose of VPP administered to male dogs (mg/kg/day)				Dose of VPP administered to female dogs (mg/kg/day)			
Organ and observed changes	0	2	8	16	0	2	8	16
Heart								
Valvular hemocyst	1/5 (20%)	3/5 (60%)	0/5	2/5 (40%)	0/5	1/5 (20%)	0/5	0/5
Kidneys								
Papillary mineralization	4/5 (80%)	4/5 (80%)	5/5 (100%)	4/5 (80%)	5/5 (100%)	4/5 (80%)	5/5 (100%)	5/5 (100%)
Cytoplasmic vacuolation, proximal convoluted tubules	2/5 (40%)	3/5 (60%)	2/5 (40%)	2/5 (40%)	4/5 (80%)	3/5 (60%)	1/5 (20%)	4/5 (80%)
Ovaries immature	—	—	—	—	5/5 (100%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
Thymus								
Involution/atrophy	1/5 (20%)	1/5 (20%)	3/5 (60%)	3/5 (60%)	2/5 (40%)	1/5 (20%)	0/5	1/5 (20%)
Thyroids								
Ectopic thymic tissue	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5 (20%)
Focal parafollicular cell hyperplasia	0/5	1/5 (20%)	0/5	0/5	1/5 (20%)	0/5	0/5	1/5 (20%)
Interstitial lymphoid infiltration	0/5	0/5	1/5 (20%)	0/5	0/5	0/5	0/5	0/5
Follicular cyst(s)	0/5	0/5	0/5	0/5	0/5	1/5 (20%)	0/5	0/5

[a]Data expressed as incidence of change/number of animals examined microscopically.

Eight-Week Study in Dogs

Gavage administration of VPP in gelatin capsules daily for 8 weeks at a top dose of 16 mg/kg/day produced no evidence of physical, biochemical, or histopathological changes associated with the administration of the test article. Statistically significant variations in hematologic and biochemical parameters were small, lacked relationships to doses and were generally observed in only a single sex.

A single, statistically significant difference was identified in the clinical pathology parameters; it was a decrease in mean neutrophil counts in all dosed female groups. This finding appears to be related to an increase in the control values in the absence of a decrease in the dosed animals. Specifically, there was a 36% increase in mean control values at week 7 compared to the pretreatment period, whereas, for the dosed animals, there was no change in the values.

A single statistically significant difference was observed in the organ weight parameters. Mean ovarian weight was significantly elevated in dogs administered 16 mg/kg/day of VPP (approximately 35%). This parameter was increased, but not statistically significant, in a non–dose-response manner in the low- and mid-dose groups (28% and 13%, respectively). In the absence of corroborating histopathology findings and based upon the findings in the low-, medium-, and high-dose groups, the authors concluded that the observed change in uterine weight was likely to be normal background variation.

Histopathologic examinations of tissues from dogs revealed only variations that occurred in control as well as dosed groups. The changes were generally graded as being minimal to slight in severity and there was no evidence to associate any changes with administration of VPP.

The results of these studies expand upon and are consistent with previously published findings on the safety of VPP and IPP. Substances containing various levels of VPP and IPP, and VPP itself, have been evaluated in short term studies (Maeno et al. 2005b). Powdered *L. helveticus*–fermented milk (4000 mg/kg body weight [BW] containing 3.7 mg/kg BW of VPP and 2.1 mg/kg BW of IPP), pasteurized casein hydrolysate (2000 mg/kg BW containing 6.0 mg/kg BW of VPP and 5.9 mg/kg BW of IPP), and synthesized VPP (up to 400 mg/kg BW) were administered to rats by single oral gavage and the rats observed for 14 days. No treatment regimen resulted in either antemortem or postmortem evidence of systemic or local toxicity. In a repeated-dose study, powdered *L. helveticus*–fermented milk was administered by gavage, up to 2000 mg/kg BW/day (containing 1.9 mg/kg BW/day of VPP and 1.1 mg/kg BW/day of IPP), to rats for 28 consecutive days. There was neither in-life nor postmortem evidence that administration of powdered *L. helveticus*–fermented milk caused either physiological or toxicological changes (Maeno et al. 2005b).

Additional studies were performed and have been reported in other publications in this issue of the *International Journal of Toxicology*. These include a 13-week repeated-dose study in rats (Mizuno et al. 2005), reproductive toxicity studies in rats (Kurosaki et al. 2005), and more specialized safety testing, including the micronucleus test in rats and mice (Matsuura et al. 2005), an evaluation of cytotoxicity and clastogenicity (Maeno et al. 2005a), and the *Salmonella—Scherichia coli*/microsome incorporation assay (Bernard et al. 2005a).

CONCLUSIONS

The oral administration of up to 16 mg/kg/day of VPP to male and female rats and dogs for 56 consecutive days produced no physical, biochemical, neurological, or pathological evidence of treatment-associated changes. Statistically significant variations in hematologic and biochemical parameters were small, lacked relationships to doses, and were generally observed in one sex, but not the other.

The results of the studies reported herein support a conclusion that the no-observable-effect level (NOEL) and maximally tolerated dose (MTD) for VPP administered to both rats and dogs continuously for 8 weeks are $\geq$16 mg/kg/day. Based upon current projections of VPP incorporation into food, this equates to a margin of safety (MOS) of at least 160.

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