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Comparative Subchronic Studies on 2,4-Dichlorophenoxyacetic Acid, Amine, and Ester in Rats[1]

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Forms of 2,4-dichlorophenoxyacetic acid (collectively known as 2,4-D) are herbicides used to control a wide variety of broadleaf and woody plants. Subchronic toxicity studies in rats were conducted on three forms of 2,4-D: the parent form, 2,4-D acid; 2,4-D dimethylamine salt (DMA); and 2,4-D 2-ethylhexyl ester (2-EHE). Doses in the subchronic studies (on an acid equivalent basis) were 0, 1, 15, 100, and 300 mg/kg/day. Major treatment related findings in the three studies included decreases in red cell mass, decreases in T3 and T4 levels, decreases in ovary and testes weights, increases in liver, kidney, and thyroid weights, and cataracts and retinal degeneration (high-dose females). These data demonstrated the comparable toxicities of 2,4-D acid, DMA, and 2-EHE and support a subchronic no-observed-effect level of 15 mg/kg/day for all three forms. In summary, the findings of these studies indicate comparable low subchronic toxicity potentials among representative forms of 2,4-D.

Forms of 2,4-dichlorophenoxyacetic acid (collectively known as 2,4-D) were some of the first herbicides to be registered in the United States and have been widely used in the control of broadleaf and woody plants on rangelands, lawns, golf courses, forests, roadways, parks, and agricultural land. 2,4-D is used extensively because of its efficacy and low acute toxicity. The various forms of 2,4-D are absorbed through both the roots and leaves of most plants, especially broadleaf species (EPA, 1989). 2,4-D's structure is similar to that of the plant-specific hormone indole acetic acid and thus acts as a plant growth regulator. The acid is the parent compound, but many of the 2,4-D formulations in use contain the amine salts, which are more water soluble than the acid, or the ester derivatives, which are readily dissolved in an organic solvent. 2,4-D is regarded as having low potential for mammalian toxicity (Munro *et al.,* 1992).

Although there was a previous 2,4-D acid subchronic rat study (Gorzinski *et al.,* 1987), it only investigated the parent compound and was conducted at a maximum dose of 150 mg/kg/day. Therefore, to enhance the 2,4-D toxicology data base, subchronic toxicity studies in rats were conducted on three representative forms of 2,4-D: 2,4-D acid; 2,4-D dimethylamine salt (DMA); and 2,4-D 2-ethylhexyl ester (2-EHE). The studies were designed to elucidate the comparative toxicities of the three forms and investigate effects at higher dose levels. All studies were conducted in accordance with Good Laboratory Practice regulations and applicable toxicology guidelines for pesticide testing (EPA, 1984; EPA-FIFRA, 1990; MAFF, 1985; EEC, 1987, 1988).

MATERIALS AND METHODS

Test chemicals and treatments. All test chemicals were obtained from the Industry Task Force II on 2,4-D Research Data. The 2,4-D acid was the technical grade with a purity of 96.4%. The DMA test substance was a 66.2% technical grade aqueous solution (95.3% purity based on dry weight), and 2-EHE had a purity of 95.1%. The analytical method utilized for test chemicals was high-pressure liquid chromatography. For all studies, the test chemicals were admixed in commercially available Purina Certified Rodent Chow 5002 (weekly) and were available *ad libitum.* At each diet mixing, dietary levels were adjusted based on the most recent body weight and food consumption determinations in order to deliver a constant average dietary intake (mg/kg body wt). 2,4-D acid and 2-EHE were added directly to the diet without any other vehicle. Acetone was utilized as the carrier for DMA and was also added to the control Purina Chow in the DMA study.

Homogeneity and stability of the test chemicals in the diets were verified analytically by gas chromatography for all studies. Concentration analyses of the dietary formulations for each group were determined weekly until termination. These analyses indicated that all diets were homogeneous and within ±10% of targeted concentrations. Test chemical intakes were generally within ±10% of targeted doses, as calculated from the diet concentration analyses, the actual food consumption, and body weight measurements in the studies.

The 13-week subchronic studies were conducted at Hazleton Washington (Vienna, VA) and are reported herein. There were 10 rats/sex/group. Target

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TABLE 1
Key Results of the 13-Week Study in F344 Rats on 2,4-D Acid[a]

	mg/kg/day				
	Control	1	15	100	300
Males					
Survival	10/10	10/10	9/10	10/10	10/10
Body weight gain (g)	194 ± 18	194 ± 11	179 ± 22	177 ± 10*	122 ± 20*
RBC counts (millions/μl)	9.7 ± 0.1	9.6 ± 0.1	9.7 ± 0.2	9.8 ± 0.2	8.6 ± 0.1*
Hemoglobin (g/dl)	17.2 ± 0.3	17.1 ± 0.2	17.2 ± 0.5	17.3 ± 0.5	16.5 ± 0.3*
Platelet counts (thousands/μl)	839 ± 33	831 ± 26	753 ± 218	792 ± 38*	676 ± 28*
T3 levels (ng/dl)	76.9 ± 10.7	69.3 ± 9.8	80.7 ± 13.0	77.4 ± 25.1	56.3 ± 8.2*
T4 levels (μg/dl)	3.9 ± 0.5	3.6 ± 0.3	3.7 ± 0.3	2.9 ± 0.6*	1.0 ± 0.0*
Thyroid/body weight ratio	0.007 ± 0.001	0.008 ± 0.001	0.008 ± 0.001	0.009 ± 0.001*	0.013 ± 0.002*
Testes/body weight ratio	1.43 ± 0.07	1.44 ± 0.05	1.47 ± 0.07	1.49 ± 0.07	0.95 ± 0.24*
Kidney/body weight ratio	0.69 ± 0.03	0.69 ± 0.02	0.74 ± 0.04*	0.84 ± 0.06*	0.83 ± 0.06*
Liver/body weight ratio	2.57 ± 0.09	2.60 ± 0.13	2.64 ± 0.25	2.83 ± 0.30*	3.67 ± 0.50*
Hypertrophy of adrenal cortex	0/10	0/10	0/10	1/10	8/10
Centrilobular hepatocellular hypertrophy	0/10	0/10	0/10	3/10	7/10
Females					
Survival	10/10	9/10	10/10	10/10	10/10
Body weight gain (g)	84 ± 8	84 ± 8	82 ± 7	74 ± 9	36 ± 13*
RBC counts (millions/μl)	9.0 ± 0.2	9.0 ± 0.2	9.1 ± 0.2	8.8 ± 0.2	7.8 ± 0.3*
Hemoglobin (g/dl)	17.0 ± 0.5	17.0 ± 0.4	16.9 ± 0.4	16.7 ± 0.3	15.4 ± 0.8*
Platelet counts (thousands/μl)	852 ± 46	862 ± 48	804 ± 152	708 ± 122*	667 ± 48*
T3 levels (ng/dl)	87.6 ± 9.7	96.0 ± 19.4	99.3 ± 17.4	65.7 ± 12.7*	57.6 ± 9.7*
T4 levels (μg/dl)	2.4 ± 0.5	2.6 ± 0.8	2.1 ± 0.5	1.0 ± 0.0*	1.0 ± 0.0*
Thyroid/body weight ratio	0.013 ± 0.004	0.013 ± 0.003	0.012 ± 0.002	0.014 ± 0.002	0.021 ± 0.004*
Kidney/body weight ratio	0.76 ± 0.03	0.76 ± 0.05	0.79 ± 0.05	0.85 ± 0.05*	1.00 ± 0.09*
Liver/body weight ratio	3.13 ± 0.49	2.96 ± 0.34	3.05 ± 0.40	3.23 ± 0.49	4.82 ± 0.89*
Cataracts	0/10	0/10	0/10	0/10	5/10
Hypertrophy of adrenal cortex	0/10	0/10	0/10	10/10	10/10
Centrilobular hepatocellular hypertrophy	0/10	0/10	0/10	0/10	9/10

[a] Values reported are at the interval before study termination.
* Significantly different from control value, $p \leq 0.05$.

doses (on an acid equivalent basis) were 0, 1, 15, 100, and 300 mg/kg/day. These doses were selected based on the results from previously conducted short-term testing (Gorzinski *et al.,* 1987).

Laboratory animals and care. Male and female Fischer 344 rats (4 weeks old) were obtained from Charles River Laboratories (Raleigh, NC). Rats were selected for the studies based on examination by a veterinarian after an acclimation period of at least 7 days. A weight randomization computer program designed to ensure homogeneity of body weights was used to select and assign the animals to the experimental groups. The animals were individually housed in elevated stainless steel, wire-mesh cages. A 12-hr light/dark cycle was maintained in the room and food and water were available *ad libitum.*

For all studies, animals were observed for overt toxicity, moribundity, and mortality at least twice daily. Animal weights, detailed clinical observations, and food consumption were determined weekly. Ophthalmoscopic examinations of all rats and mice were conducted prior to treatment and at study termination (Week 13).

Clinical pathology. Clinical chemistry, hematology, and urinalyses were performed during Weeks 6 and 13. Blood samples were collected by orbital sinus venipuncture under ketamine hydrochloride anesthesia following an overnight fast. Urine samples were collected from fasted animals overnight in urine collection cages.

Hematology parameters evaluated included cell morphology, corrected leukocyte count, erythrocyte count, hematocrit, hemoglobin, leukocyte count, leukocyte differential, and platelet count. Serum chemistry parameters investigated included alanine aminotransferase (ALT), albumin, aspartate aminotransferase (AST), blood urea nitrogen (BUN), lactate dehydrogenase (LDH), alkaline phosphatase, calcium, chloride, creatinine, globulin, glucose, inorganic phosphorus, potassium, sodium, total bilirubin, total cholesterol, total protein, triiodothyronine (T3), and thyroxine (T4). Urinalysis measurements included bilirubin, glucose, ketones, pH, protein, specific gravity, and urobilinogen.

Anatomic pathology. All animals surviving to study termination (Week 13) were anesthetized by sodium pentobarbital, exsanguinated, and subjected to gross and microscopic examinations. A complete necropsy was performed on all animals. The following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries, pituitary, testes (with epididymides), thymus, and thyroid/parathyroids. The following tissues from each animal were preserved in 10% neutral-buffered Formalin: adrenals, aorta, bone marrow (sternum), brain with brain stem, cecum, esophagus, eyes (fixed in Bouin's and stored in 70% alcohol), femur, heart, kidneys, duodenum, jejunum, ileum, colon, rectum, lacrimal gland, liver, lung, mammary gland, mesenteric lymph nodes, ovaries, pancreas, pituitary, salivary gland, sciatic nerve, skeletal muscle, spinal cord (three levels), spleen, stomach, testes

TABLE 2
Key Results of the 13-Week Study in F344 Rats on 2,4-D DMA[a]

	mg/kg/day (acid equivalents)				
	Control	1	15	100	300
Males					
Survival	10/10	10/10	10/10	10/10	10/10
Body weight gain (g)	173 ± 12	168 ± 18	172 ± 11	173 ± 15	100 ± 15*
RBC counts (millions/μl)	9.6 ± 0.2	9.6 ± 0.1	9.5 ± 0.3	9.5 ± 0.1	8.5 ± 0.1*
Hemoglobin (g/dl)	16.9 ± 0.4	16.9 ± 0.2	16.8 ± 0.4	16.8 ± 0.3	16.4 ± 0.2*
Platelet counts (thousands/μl)	854 ± 37	813 ± 138	833 ± 39	854 ± 90	708 ± 28*
T3 levels (ng/dl)	85.9 ± 21.7	99.9 ± 28.0	97.4 ± 16.4	89.4 ± 18.3	75.3 ± 17.8
T4 levels (μg/dl)	3.9 ± 0.6	4.5 ± 0.5	4.2 ± 0.7	3.4 ± 0.6	1.0 ± 0.1*
Thyroid/body weight ratio	0.005 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.008 ± 0.002*
Testes/body weight ratio	1.41 ± 0.05	1.39 ± 0.05	1.42 ± 0.06	1.42 ± 0.11	0.80 ± 0.08*
Kidney/body weight ratio	0.67 ± 0.04	0.67 ± 0.03	0.71 ± 0.02	0.75 ± 0.03*	0.77 ± 0.03*
Liver/body weight ratio	2.49 ± 0.16	2.51 ± 0.14	2.58 ± 0.16	2.58 ± 0.13	3.06 ± 0.25*
Brush border loss of tubular cells	0/10	0/10	0/10	0/10	7/10
Hypertrophy of adrenal cortex	0/10	0/10	0/10	0/10	10/10
Centrilobular hepatocellular hypertrophy	0/10	0/10	0/10	0/10	0/10
Females					
Survival	10/10	10/10	10/10	10/10	9/10
Body weight gain (g)	70 ± 9	75 ± 11	70 ± 6	61 ± 6	24 ± 14*
RBC counts (millions/μl)	9.0 ± 0.2	8.8 ± 0.2	9.0 ± 0.2	8.6 ± 0.1*	7.8 ± 0.5*
Hemoglobin (g/dl)	16.7 ± 0.4	16.4 ± 0.4	16.7 ± 0.3	16.3 ± 0.3	15.4 ± 1.1*
Platelet counts (thousands/μl)	838 ± 62	882 ± 33	882 ± 36	705 ± 52*	669 ± 31*
BUN (mg/dl)	17 ± 1.6	17 ± 1.1	17 ± 1.1	16 ± 1.7	21 ± 3.1*
T3 levels (ng/dl)	96.8 ± 14.9	92.9 ± 17.4	94.9 ± 11.7	75.7 ± 12.7*	71.1 ± 19.8*
T4 levels (μg/dl)	2.7 ± 0.6	2.4 ± 0.5	2.5 ± 0.6	0.9 ± 0.1*	0.9 ± 0.0*
Thyroid/body weight ratio	0.006 ± 0.001	0.008 ± 0.001	0.007 ± 0.003	0.008 ± 0.002	0.012 ± 0.003*
Kidney/body weight ratio	0.75 ± 0.05	0.73 ± 0.03	0.74 ± 0.05	0.80 ± 0.03	0.93 ± 0.10*
Liver/body weight ratio	2.72 ± 0.11	2.77 ± 0.10	2.73 ± 0.13	2.80 ± 0.11	4.10 ± 0.69*
Cataracts	0/10	0/10	0/10	0/10	7/10
Retinal degeneration	0/10	0/10	0/10	0/10	6/10
Hypertrophy of adrenal cortex	0/10	0/10	0/10	0/10	9/10
Brush border loss of tubular cells	0/10	0/10	0/10	0/10	8/10
Centrilobular hepatocellular hypertrophy	0/10	0/10	0/10	0/10	8/10

[a] Values reported are at the interval before study termination.
* Significantly different from control value, $p \leq 0.05$.

with epididymides, thymus, thyroid/parathyroids, trachea, urinary bladder, uterus, and any other tissues with gross lesions. Preserved tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically.

Statistics. Rats selected for the studies were stratified by weight and randomly assigned to treatment groups using a computerized randomization program. Bartlett's test, performed following randomization, ensured homogeneity of body weight variances and means (Bartlett, 1937). Body weight changes, total feed consumption, clinical pathology data (except cell morphology findings and routine urinalysis data), and organ weight data of the control group were compared statistically to the data from the same sex and interval of the treated groups using Analysis of Variance (ANOVA) (Winer, 1971). If variances of untransformed data were heterogeneous, a series of transformations was performed in an effort to achieve variance homogeneity using Levene's test (Draper and Hunter, 1969; Levene, 1960). When the series of transformations was not successful in achieving variance homogeneity, analyses were performed on rank-transformed data. Group comparisons were routinely performed using Dunnett's t test at the 5% two-tailed probability level (Dunnett, 1955, 1964).

RESULTS

The comparative toxicities of 2,4-D acid, DMA, and 2-EHE are shown in Tables 1–3. The three test chemicals were relatively well tolerated at the low- and mid-dose levels, while severe body weight gain depressions were seen at 300 mg acid equivalents/kg/day. Other than for 2-EHE at 300 mg acid equivalents/kg/day, there were no treatment-related deaths or severe illnesses.

In all three studies, the rates of body weight gain and food consumption were retarded in certain groups of treated animals of both sexes when compared with controls. Severe body weight gain depression was observed at 300 mg/kg/day in each of the three studies. For males this depression ranged from 37 to 80% of controls; for females the range was 57 to 88%. The no-observed-effect level (NOEL) for

TABLE 3
Key Results of the 13-Week Study in F344 Rats on 2,4-D 2-EHE[a]

	mg/kg/day (acid equivalents)				
	Control	1	15	100	300
Males					
Survival	10/10	10/10	10/10	10/10	5/10
Body weight gain (g)	159 ± 17	143 ± 17	164 ± 16	150 ± 11	33 ± 23*
RBC counts (millions/μl)	9.6 ± 0.2	9.7 ± 0.3	9.7 ± 0.3	9.7 ± 0.2	7.4 ± 0.4*
Hemoglobin (g/dl)	16.7 ± 0.5	16.9 ± 0.5	16.8 ± 0.5	16.9 ± 0.3	13.8 ± 0.8*
Platelet counts (thousands/μl)	897 ± 50	865 ± 66	916 ± 20	824 ± 38*	694 ± 57*
BUN (mg/dl)	16 ± 1.6	15 ± 2.0	16 ± 1.3	14 ± 2.0	22 ± 3.7*
T4 levels (μg/dl)	3.8 ± 0.7	3.9 ± 0.6	3.7 ± 0.5	3.5 ± 0.5	0.9 ± 0.0*
Thyroid/body weight ratio	0.006 ± 0.001	0.007 ± 0.001	0.007 ± 0.002	0.007 ± 0.002	0.020 ± 0.002*
Testes/body weight ratio	1.52 ± 0.07	1.57 ± 0.11	1.64 ± 0.37	1.54 ± 0.12	0.93 ± 0.15*
Kidney/body weight ratio	0.72 ± 0.06	0.74 ± 0.05	0.82 ± 0.14	0.85 ± 0.5*	0.98 ± 0.7*
Liver/body weight ratio	2.57 ± 0.8	2.61 ± 0.29	2.90 ± 0.49	2.61 ± 0.15	5.78 ± 0.57*
Vacuolization of tubular cells	0/10	0/10	0/10	0/10	5/10
Hypertrophy of adrenal cortex	0/10	0/10	0/10	0/10	6/10
Centrilobular hepatocellular hypertrophy	0/10	0/10	0/10	0/10	6/10
Females					
Survival	10/10	10/10	10/10	10/10	7/10
Body weight gain (g)	68 ± 8	73 ± 6	72 ± 9	64 ± 8	8 ± 8*
RBC counts (millions/μl)	9.0 ± 0.2	9.0 ± 0.2	9.1 ± 0.2	8.7 ± 0.2*	7.7 ± 0.3*
Hemoglobin (g/dl)	16.7 ± 0.2	16.7 ± 0.4	16.8 ± 0.5	16.4 ± 0.6	14.6 ± 0.8*
Platelet counts (thousands/μl)	867 ± 79	910 ± 44	889 ± 53	725 ± 68*	643 ± 68*
BUN (mg/dl)	17 ± 1.6	17 ± 1.6	18 ± 1.5	16 ± 1.1	26 ± 2.7*
T 4 Levels (μg/dl)	2.3 ± 0.7	2.0 ± 0.6	2.1 ± 0.8	0.9 ± 0.0*	0.9 ± 0.0*
Thyroid/body weight ratio	0.015 ± 0.007	0.017 ± 0.006	0.015 ± 0.003	0.016 ± 0.004	0.024 ± 0.004*
Kidney/body weight ratio	0.82 ± 0.10	0.78 ± 0.04	0.88 ± 0.16	0.89 ± 0.11	1.15 ± 0.10*
Liver/body weight ratio	3.06 ± 0.46	2.86 ± 0.20	3.07 ± 0.52	3.13 ± 0.35	6.00 ± 0.59*
Cataracts	0/10	0/10	0/10	0/10	10/10
Retinal degeneration	0/10	0/10	0/10	0/10	6/10
Hypertrophy of adrenal cortex	0/10	0/10	0/10	0/10	10/10
Vacuolization of tubular cells	0/10	0/10	0/10	0/10	6/10
Centrilobular hepatocellular hypertrophy	0/10	0/10	0/10	0/10	8/10

[a] Values reported are at the interval before study termination.
* Significantly different from control value, $p \leq 0.05$.

body weight and food consumption effects for the DMA and 2-EHE studies was considered to be 100 mg/kg/day. A NOEL of 15 mg/kg/day was established for these parameters in the 2,4-D acid study due to body weight gain effects noted in males at 100 mg/kg/day.

Changes in red cell mass, platelet count, T3, and T4 were seen consistently among the three studies. The NOEL for hematology and clinical chemistry effects in all three studies was 15 mg acid equivalents/kg/day.

With respect to organ weights, effects were consistently seen in all three studies for liver, kidney, testes, and thyroid. The statistically significant increase in kidney/body weight in males of the 15 mg/kg/day 2,4-D acid group was not considered to be toxicologically significant since it fell within historical control ranges and the incidence of the effect was low in absolute numbers. The NOEL for organ weight effects was 15 mg acid equivalents/kg/day.

Histological alterations in the three studies were seen predominantly at the 300 mg acid equivalents/kg/day groups and consisted of retinal degeneration and cataract formation (females), centrilobular hepatocellular hypertrophy (both sexes), atrophy of the testes, hypertrophy in the zona glomerulosa of the adrenal cortex (both sexes), brush border loss in proximal tubular cells in the kidney (both sexes), and vacuolization of kidney tubular cells (both sexes). The effects noted in the liver, testes, kidney, and adrenal were considered to be of a slight degree and occurred at a dose that clearly exceeded the maximum tolerated dose (MTD). The overall NOEL for each of the three subchronic studies following histological evaluation was considered to be 15 mg acid equivalents/kg/day.

DISCUSSION

The data from the three studies build a strong case for comparable toxicities of 2,4-D acid, DMA, and 2-EHE. The results of these studies are in agreement with those reported by Gorzinski *et al.* (1987). Significantly, the current studies noted cataracts and retinal degeneration only at 300 mg/kg/day. Gorzinski *et al.* did not report any adverse ophthalmic findings at doses up to 150 mg/kg/day, which was also the case at 100 mg/kg/day in the current studies. Subchronic eye effects are clearly unique to a high-dose treatment.

The similarity of the toxic responses seen in the 2,4-D acid and 2-EHE studies is additionally consistent with findings from rat pharmacokinetic studies which predicted comparable toxicities of these two forms of 2,4-D due to the demonstrated rapid conversion of 2-EHE to 2,4-D acid on oral treatment (Frantz and Kropscott, 1993). DMA toxicity was of the same magnitude as that of the other two forms, presumably due to physiological dissociation of DMA to 2,4-D acid. Additionally, the rat data are consistent with the findings from a series of subchronic dog studies in which comparable toxicities of the same forms of 2,4-D were also demonstrated (Charles *et al.,* 1996a).

The dose of 300 mg/kg/day in each of the studies clearly exceeded the MTD based on the severe body weight gain depression versus controls. The next lower dose of 100 mg/kg/day did not exceed the MTD.

The overall NOEL for the combined subchronic studies was 15 mg acid equivalents/kg/day. This is equivalent to the NOEL identified in previous work (Gorzinski *et al.,* 1987). It is recognized that subchronic toxicity studies are good indicators of chronic toxicity. In support of this, the 1986 two-year chronic/oncogenicity rat study identified a 5 mg/kg/day NOEL (Serota, 1986). In a recently conducted two-year chronic/oncogenicity study, a NOEL of 5 mg/kg/day was also established (Charles *et al.,* 1996b).

In conclusion, 2,4-D acid and its forms have been tested in rodents over a wide range of dose levels, including high doses that severely compromise animal homeostasis. The findings of these studies indicate comparable subchronic toxicities among representative forms of 2,4-D. An overall NOEL of 15 mg/kg/day for subchronic effects was established.

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