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SUBCHRONIC ORAL TOXICITY, TISSUE DISTRIBUTION AND CLEARANCE OF HEXACHLOROETHANE IN THE RAT

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ABSTRACT

Hexachloroethane (HCE) was fed to Fischer 344 rats at approximate doses of 0, 1, 15 or 62 mg/kg/day for 16 weeks. Selected tissues were assayed at termination for HCE content. Histopathological examination identified the kidney as the primary target organ with male rats more sensitive than female rats. The kidney concentration of HCE increased proportionately with dose in the males, but there were disproportionately small increases with dose in females. A group of male rats was given 62 mg/kg/day for 8 weeks to estimate tissue clearance. Clearance of HCE from fat, liver, kidney and blood occurred in an apparent first-order manner with a half-life of approximately 2.5 days. The apparent first-order elimination suggests that HCE metabolism and excretion were not saturated in rats given up to 62 mg/kg/day and suggests that, in the range of doses given, toxicity should be proportional to exposure concentration. The no-observable-effect level (NOEL) for toxicity was 1 mg/kg/day for male and female rats.

INTRODUCTION

Hexachloroethane (HCE), a white crystalline solid that sublimes, has been used as a chemical intermediate in the manufacture of pyrotechnics, chlorinated materials, insecticides and parasiticides [1,2]. Single dose oral LD50 values in rats have been reported to be from 4500 to 7700 mg/kg [3,4].

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Rats exposed by inhalation to 260 ppm, 6 hours a day, 5 days a week, for 6 weeks showed tremors and a red exudate around the eyes after 4 weeks. The relative kidney, spleen and testes weights of male rats and the relative liver weight of female rats were increased at six weeks. No untoward effects were seen in an adjunct group of rats exposed for 6 weeks and allowed to recover for 12 weeks. Rats exposed to 15 or 48 ppm did not exhibit any treatment-related effects [4]. Gavage administration of 590 and 1179 mg/kg/day of HCE to male and female B6C3F1 mice for 78 weeks was reported to increase the incidence of hepatocellular carcinomas whereas no increase in tumor incidence was shown for either sex of Osborne-Mendel rats given 212 or 423 mg/kg/day for 66 of 78 weeks [5]. Tubular nephropathy was noted in approximately 60% of male and female rats given the high dose, and also in female (18%) and male (45%) rats fed the low dose (loc. cit.).

Data were not available on the potential toxicity of HCE when administered via the diet; this route was considered to reflect normal ingestion and is preferable to gavage administration. The results of a subchronic (16-week) dietary toxicity study, with data on tissue distribution and clearance are reported herein.

MATERIALS AND METHODS

Test Material and Diet Preparation

The HCE sample was obtained from Eastman Kodak Co., Rochester, NY. Gas chromatographic analysis determined the purity to be 99.4%.

Test diets were prepared every 2 to 3 days using Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO). A feed concentrate of HCE was serially diluted to prepare the lower dose levels. To minimize postmixing loss (due to sublimination) of HCE from the feed, test diets were stored in covered glass jars following preparation. Feed crocks (o.d., 7 cm; h, 6 cm) were filled daily in the afternoon (about 4 p.m.) with fresh diets from the stock jars. The crocks were removed the following morning

(about 7 a.m.), at which time the feed in the crocks was discarded. Thus, the rats had access to the treated food for approximately 15 hrs per day.

Diurnal Eating Pattern

Since HCE is volatile and could also act as an aversive adulterant of food, there was a possibility that the rats would delay eating until the HCE levels had declined to a "tolerable" level. To test for this possibility, four Fischer 344 female rats were placed in cages (one per cage) and eating behavior was monitored by utilization of a timer that was controlled by an infrared photocell. The photobeam crossed the top of the food crock. Concentration of HCE in food was approximately 1.2 mg/g when put into the crocks (time = 0). This HCE content was the same as for the high dose group in the main portion of the dietary study.

The daily distribution of eating time was estimated by logging the cumulative amount of time the photobeam was broken during consecutive 2-hr intervals. There were twelve 2-hr intervals a day, and the timing 'day' began at 4 p.m. Lights were turned off at 6 p.m. and turned on at 6 a.m. Data were collected for four days. Untreated food was presented at 5 p.m. on the first and fourth days, and HCE treated food was presented at 5 p.m. on the second and third days. To test for effects of removing the food during the daytime, food crocks were left in the cages during the first and second day, and were removed at 7 a.m. on the third and fourth days.

Experimental Design

Male (260-300 grams) and female (150-175 grams) CDF Fischer 344 rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were used for the study. The animals were randomly divided into dose groups of 10 per sex and were fed 0 (control), 1, 15 or 62 mg HCE/kg body weight/day. (Targeted dose levels were 3, 30 and 100 mg/kg/day, however, loss of HCE during diet preparation

resulted in the 1, 15 and 62 mg/kg/day dose levels). An additional group of 20 male rats was fed the highest dose to obtain data on tissue distribution and clearance. The rats were housed singly in stainless steel cages in a room designed for controlled humidity, temperature, 12-hr photocycle, and approximately 10 air exchanges per hour.

Body weights were recorded weekly, and food consumption was measured at least once and usually twice a week (nonconsecutive days). Routine urinalyses and hematologic determinations were conducted during the 13th week of the study. Hematologic parameters measured (Coulter Counter ZBI and Hemoglobinometer, Coulter Electronics, Hialeah, FL) were packed cell volume (PCV) (Microhematocrit Centrifuge, Clay-Adams Co., N.Y.), erythrocyte count (RBC), hemoglobin concentration (Hgb) and total and differential leukocyte counts (WBC). Routine urinalyses included measurement of specific gravity (T. S. Meter, American Optical Co., Keene, NH) and semiquantitative estimates (Multistix-Ames Company, Elkhart, IN) of urinary pH, glucose, protein, ketones, occult blood and urobilinogen. Clinical biochemical determinations (CentrifiChem System 400, Union Carbide Corp., Rye, NY) of serum urea nitrogen (BUN), creatinine and activities of glutamic-pyruvic transaminase (SGPT) and alkaline phosphatase (AP) were made on serum obtained at necropsy after 16 weeks on test.

At necropsy, each rat was examined grossly and the brain, heart, liver, kidneys and testes were excised and weighed. Representative portions of the following organs and tissues from each rat were preserved in phosphate-buffered 10% formalin: esophagus, salivary gland, stomach, small intestine, large intestine, cecum, pancreas, liver, kidneys, urinary bladder, prostate, seminal vesicles, coagulating gland, testes, epididymis, ovaries, uterus, mammary gland, brain (cerebellum, cerebrum, brain stem), spinal cord, pituitary gland, peripheral nerve (sciatic), trachea (larynx), lungs (bronchi), sternum, spleen, thymus, lymph nodes (thoracic, mesenteric), heart, aorta, skeletal muscle, adrenal glands, thyroid gland, parathyroid glands, mesenteric

adipose tissue, skin, tongue, any gross lesion or mass. The eyes of 5 rats/sex/dose were preserved in Zenker's fixative and those of the remaining 5 rats/sex/dose were preserved in formalin. Hematoxylin and eosin-stained sections were processed and examined from the tissues listed above, except sternum, of all rats in the control and highest dose group. Sections of liver and kidneys from both sexes and adrenals from males were similarly processed and examined from rats in the lower two dose groups.

Tissue Clearance and Distribution

A group of 20 male rats was given the high dose diet for 8 weeks, and then placed on untreated control diets. Three to four of these rats were sacrificed after 3, 6, 13, 22 and 31 days on control feed to obtain samples of fat, liver, kidney and whole blood for analysis of HCE. Samples of the above tissues were also taken for HCE analysis from 4 rats/ sex/dose that were necropsied at termination of the 16-week subchronic toxicity study. Analyses were conducted on hexane extracts of the tissues by GLC using a Varian (Varian Assoc., Walnut Creek, CA) 3700 gas chromatograph (GC) equipped with an electron capture detector, and a glass column (7 ft x 2 mm ID) packed with 10% OV-11 on 100/120 mesh Supelcoport (Supelco Inc., Bellefonte, PA). Area responses for the GC peaks were obtained using a Varian CDS 111 integrator. The extraction efficiencies for HCE from blood, liver, kidney and fat into hexane were determined to be 98.6%, 82.9%, 100% and 71%, respectively. The limit of detection was 0.001 μg/g. Half-lives for tissue clearance were obtained by linear least-square analysis [6].

Data Analysis

Data from the subchronic toxicity study were examined statistically by a one-way analysis of variance; differences between treated and control groups were identified using Dunnett's test [7]. The level of significance is reported as $\alpha = 0.05$ for all cases, while recognizing that the repetitive use of ANOVA on several test parameters from the same group of rats causes an

increased but undefined probability of false positive results. Thus, these statistical analyses are descriptive in nature and should be carefully interpreted.

RESULTS

Diet Analysis

Assay of the top 0.5 cm of meal revealed an HCE loss of about 10% in 2.5 hrs and about 40% in 12 hrs. Aliquots below the 0.5 cm level were assayed up to 48 hours and found to be comparable to initial concentration. Since rats eat deeper into the meal than 0.5 cm, the actual amount of HCE lost would have been somewhat less than 40% for the total amount of food consumed. The rate of food consumption during the night was shown to be reasonably linear (Figure 1) so that estimates of daily dose were calculated from measurements of food consumption and average HCE content of food. As a result, treatment doses were conservatively estimated to be 0, 1, 15 or 62 mg/kg/day (Table 1) rather than the targeted 0, 3, 30 or 100 mg/kg/day dose levels.

Diurnal Eating Pattern

A summary of the average pattern of eating is presented in Figure 1. The rats ate the HCE treated food at the same time intervals as untreated food. About 14% of eating activity occurred just after fresh crocks were placed in the cages, during the 4 to 6 p.m. interval (visual observation indicated near zero activity until the rats were disturbed by the caretakers). Most of the eating activity (78%) occurred during the 12 dark hours, and approximately 4% occurred soon after the lights came on at 6 a.m. Even when food was available between 8 a.m. and 4 p.m, minimal eating activity was recorded during these daytime hours. Thus, there was no practical difference in feeding behavior when food was withheld or available during the daytime. Because the feeding pattern was linear, the HCE daily doses were estimated from routine food consumption measurements and HCE analyses of food.

TABLE 1

Analysis for Hexachloroethane in Diets Fed to Rats on A 16-Week Subchronic Toxicity Study

Nominal Dose Level	% of Target Concentration[a]	Actual Approximate Dose (mg/kg/day)[b]
Premix (0.5%)	92±3 (4)	--
100 mg/kg/day	82±12 (8)	62
30 mg/kg/day	68±8 (4)	15
3 mg/kg/day	44±8 (6)	1.0

[a]Values are mean ± S.D. obtained from analysis of N samples; samples taken from fresh crocks (time = 0).

[b]Conservative estimate based on an average 25% additional loss over the subsequent 15 hours of daily food availability, e.g., mg/kg/day at time 0 multiplied by 0.75 = 62 mg/kg/day.

Subchronic Toxicity Evaluation

All rats survived the 16-week test period with no overt manifestations of toxicity or changes in behavior. Body-weight gains, food consumption, hematologic parameters, urinalysis and clinical biochemical determinations showed no differences between treated and control groups.

Male rats were slightly more sensitive than female rats to the nephrotoxic properties of HCE. Kidney effects in male rats were manifested by increased kidney weights (Table 2) and gross pathological alterations at the highest dose. Tubular atrophy, degeneration, hypertrophy and/or dilation were seen at the highest two doses (Table 3). Evidence of renal toxicity in females consisted of very slight renal tubular atrophy and degeneration observed histopathologically at the highest dose level (Table 3).

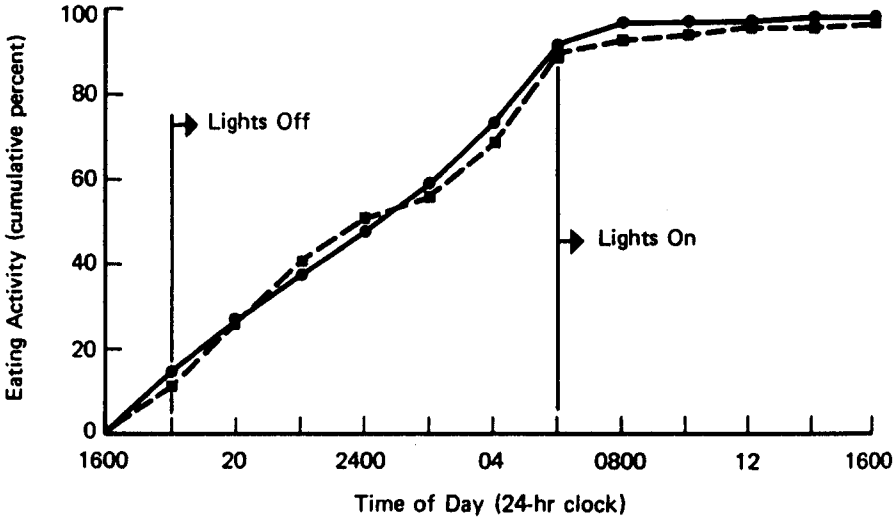

Fig. 1 Diurnal eating pattern of four female rats given control (- - -) or HCE treated (———) food (62 mg/kg/day). Each point represents average values for 2 days exposure to control or treated diets. Lines are drawn by visual inspection.

Liver weights were increased in male rats given the highest dose. The liver exhibited a slight swelling of the hepatocytes in males given the highest two doses. Female rats given the high dose had increased relative liver weight ratio unaccompanied by microscopic alterations.

All other histopathologic observations in treated rats were similar in type and severity to those seen in control rats and were considered to be spontaneous in nature and typical of those commonly observed in rats of the age and strain used in this study.

Tissue Distribution and Clearance

Figure 2 represents a semilogarithmic plot of the mean tissue concentration-time profile. The highest concentration of HCE was consistently found in the fat. Concentrations in fat were between

TABLE 2

Terminal Body, Kidney and Liver Weight Data[a] for Rats Fed Hexachloroethane in the Diet for 16 Weeks

Sex	Dose Level (mg/kg/day)	Fasted Body Weight (g)	LIVER		KIDNEYS	
			g	g/100g	g	g/100g
M	0	314.4±12.4	8.32±0.27	2.65±0.06	2.28±0.08	0.73±0.04
	1	328.0± 7.2	8.46±0.22	2.58±0.07	2.31±0.09	0.70±0.02
	15	329.0±24.4	8.69±0.80	2.64±0.09	2.40±0.15	0.73±0.01
	62	324.2±10.0	8.98±0.54[b]	2.77±0.12[b]	2.51±0.12[b]	0.77±0.02[b]
F	0	176.7± 6.9	4.65±0.26	2.63±0.06	1.40±0.08	0.79±0.03
	1	174.0± 7.9	4.74±0.22	2.73±0.11	1.38±0.05	0.79±0.03
	15	176.7± 4.6	4.79±0.21	2.69±0.09	1.39±0.06	0.79±0.04
	62	170.8± 5.1	4.71±0.23	2.76±0.10[b]	1.39±0.05	0.81±0.02

[a]Values are mean ± S.D. of 10 rats

[b]Statistically identified difference from control mean using Dunnett's test $\alpha = 0.05$.

TABLE 3

Histopathological Results[a] on Liver and Kidneys on Rats Fed Hexachloroethane in the Diet for 16 Weeks

	Sex	Dose (mg/kg/day) 0	1	15	62
Kidneys					
Slight hypertrophy and/or dilation of proximal convoluted tubules	M	0	1	7	10
	F	0	0	0	0
Atrophy and degeneration of renal tubules[b] (with a degree of peritubular fibrosis in high dose males)	M	1	2	7	10
	F	1	1	2	6
Liver					
Slight swelling of hepatocytes	M	4	3	6	8
	F	0	0	0	0

[a]Data presented as number of positive observations for 10 rats/sex/dose level.

[b]Graded as slight in 1 of 10 male control rats and very slight in 1 of 10 control female rats.

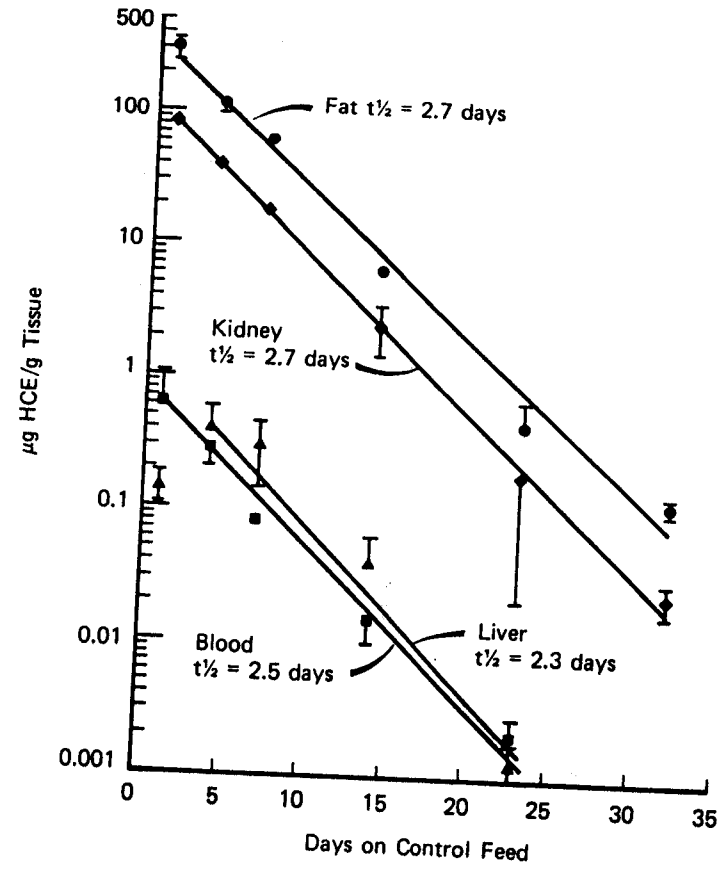

Fig. 2 Clearance of hexachloroethane (HCE) from tissues of male rats after 8 weeks on diets providing 62 mg HCE/kg/day. Each point represents the Mean ± S.D. for 3 animals. The lines drawn through the data represent the best-fit obtained by least-square linear regression analysis.

2.5 to 5 times that found in the kidney, and over 100 times the concentrations found in the liver or blood. The concentration of HCE in the fat decreased from 303±50 µg/g ($\bar{X}$±S.D.) in a first-order manner (t 1/2 = 2.7 days). The concentration of HCE in the liver, kidney and blood also decreased in an apparent first-order manner with half-lives very similar to the fat.

TABLE 4

Concentration of Hexachloroethane in the Tissues of Rats Fed Hexachloroethane in the Diet for 16 Weeks

Sex	Dose (mg/kg/day	μg HCE/g Tissue[a] Blood	Liver	Kidney	Fat
M	1.0	0.079±0.057	0.291±0.213	1.356±0.29	3.15±0.37
	15	0.596±0.653	1.736±1.100	24.33±5.73	37.90±6.10
	62	0.742±0.111	0.713±0.343	95.12±11.56	176.1±14.50
F	1.0	0.067±0.039 (3)	0.260±0.035 (2)	0.369±0.51	2.59±0.72
	15	0.162±0.049 (3)	0.472±0.204	0.688±0.17	45.27±11.33
	62	0.613±0.231	0.631±0.262	2.01±0.66	162.1±7.10

[a]Values are means ± S.D. of 4 rats except where noted in parentheses; tissues analyzed on last treatment day.

Results of analyses of tissues obtained at necropsy after 16 weeks on the test diets are summarized in Table 4. The concentration of HCE in the kidneys of male rats was higher than that of females at all dose levels. The concentration of HCE in the kidneys of the males were generally proportional to dose, while in females there was a disproportionately smaller increase in HCE levels in the kidney relative to dose. HCE content in fat was similar for male and female rats. There was a disproportionately small increase in the concentration of HCE in the liver and blood as the dose increased; this pattern was similar for both sexes.

DISCUSSION

The kidney was identified as the primary target organ in this study with male rats being more sensitive to orally ingested HCE than female rats. Minimal effects were also seen in the liver where no salient sex preference existed. A NCI carcinogenicity bioassay also found the kidneys to be the principal target organ in mice and rats [5]; their treatment regimen produced dose-related increases in mortality along with renal tubular nephropathy which was slightly higher in male rats than in female rats given the low dose of 212 mg/kg/day.

Kidneys of male rats fed HCE for 16 weeks contained HCE levels proportional to dose. In contrast, HCE levels in the kidneys from females at all doses were much less and did not increase proportionately with increasing dose. This difference in concentration may portend toxicity and contribute to the microscopic alterations seen in the kidneys.

The disproportionately small increase in the concentration of HCE in the blood and liver relative to dose may be due to saturation of protein binding sites. It appears that blood HCE concentrations did not provide a reliable estimate of HCE exposure, at least not in the rat.

The tissue clearance data indicated that HCE was eliminated by the rat with a half-life of between 2 and 3 days. Based on an

average half-life of 2.5 days, the concentration of HCE in the rat reached a plateau, i.e., 99% of steady state concentration, in 17 days. The tissue concentration data did not suggest accumulation of HCE with increasing duration of treatment.

The elimination of HCE from the rats was faster than that suggested in the literature for other species studied. Jondorf et al.[8] reported that in 3 days only 30% of the 0.5 g of ^{14}C-labeled HCE/kg given to rabbits was recovered in the urine and expired air. The remaining 70% of the dose was assumed to be in the body and led the investigators to conclude that HCE was slowly metabolized. Fowler[2] investigated the distribution of HCE in sheep following oral administration of an oil-gum emulsion. The concentration of HCE in blood peaked at 27 μg/ml 24 hours after a 0.5 g/kg dose and then remained virtually unchanged until it dropped precipitously 4 days after dosing. It is difficult to relate these results with the present study in rats which characterizes both target organ concentrations, elimination in rats and toxicity in an integrated manner.
Comparing the toxicity of HCE in the rat and rabbit, Weeks et al.[4] reported that no effects were observed in rabbits following 12 daily gavage doses of 100 mg/kg, a dose somewhat higher than the high dose fed to rats for 16 weeks in this study. This difference in response may indicate that the rat is more susceptible; however, interpretation of this difference in effects at comparable doses is not really possible due to the different durations of treatment.

Based on renal histologic changes, the no-observable-effect-level (NOEL) for toxicity for male and female rats in this study was 1 mg HCE/kg body weight/day. The apparent first-order elimination suggests that HCE metabolism and excretion were not saturated in rats receiving 62 mg/kg/day.

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