National Toxicology Program
Toxicity Report Series
Number 14

NTP Technical Report on Toxicity Studies of

p-Chloro-α,α,α-Trifluorotoluene

(CAS NO: 98-56-6)

Administered in Corn Oil and α-Cyclodextrin to F344/N Rats and B6C3F$_1$ Mice in 14-Day Comparative Gavage Studies

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NIH Publication 92-3133
July 1992

United States Department of Health and Human Services
Public Health Service
National Institutes of Health

NOTE TO THE READER

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control. In July, 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. The NTP coordinates the relevant programs, staff, and resources from the Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

The NTP develops, evaluates, and disseminates scientific information about potentially toxic and hazardous chemicals. This knowledge is used for protecting the health of the American people and for the primary prevention of disease.

The studies described in this toxicity study report were performed under the direction of the NIEHS and were conducted in compliance with NTP chemical health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals.

These studies are designed and conducted to characterize and evaluate the toxicologic potential of selected chemicals in laboratory animals (usually two species, rats and mice). Chemicals selected for NTP toxicology studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. Selection per se is not an indicator of a chemical's toxic potential. Single copies of this Report are available without charge while supplies last from the NTP Public Information Office, NIEHS, P.O. Box 12233, Research Triangle Park, NC 27709 (919-541-3991).

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CONTRIBUTORS

The NTP Report on the toxicity studies of *p*-Chloro-α,α,α-trifluorotoluene (CTFT) is based primarily on 14-day studies that began in February, 1990, and ended in March, 1990, at the National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, NC.

National Toxicology Program
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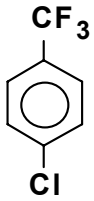
p-CHLORO-α,α,α-TRIFLUOROTOLUENE



Molecular Formula: $C_7H_4F_3Cl$

CAS No: 98-56-6

Molecular Weight: 180.6

Synonyms: CTFT; p-Chloro-4-(trifluoromethyl) benzene; (*p*-chlorophenyl) trifluoromethane; 4-chlorobenzotrifluoride; Benzene, 1-chloro-4-(trifluoromethyl)-; *p*-(trifluoromethyl) chloro-benzene; *p*-chlorobenzotrifluoride; *p*-chlorotri-fluoromethylbenzene; *p*-trifluoromethylphenyl chloride; parachloro-α,α,α-trifluorotoluene; parachlorobenzotrifluoride; parachlorotrifluoro-methylbenzene

ABSTRACT

p-Chloro-α,α,α-trifluorotoluene (CTFT) is a volatile, aromatic liquid used as a chemical intermediate in the manufacture of dinitroaniline herbicides. To evaluate the toxicity of CTFT, groups of F344/N rats and $B6C3F_1$ mice of each sex were administered CTFT by gavage once a day for 14 consecutive days in either corn oil or in an experimental molecular complex vehicle, α-cyclodextrin (α-CD). Dose levels selected for CTFT with the α-CD vehicle were 10, 50, and 400 mg/kg; dose levels used with the corn oil vehicle were 10, 50, 400, and 1000 mg/kg. The toxicokinetics of CTFT also were compared by gavage with the different vehicles and by i.v. administration. In genetic toxicity studies, CTFT was not mutagenic in *Salmonella typhimurium*.

The elimination of an intravenous dose of CTFT from blood is best described by a triexponential equation. The data best fit a 3-compartment kinetic model with a very rapid distribution phase. A biexponential equation was found to best fit the elimination of CTFT from blood following a gavage dose in either corn oil or an aqueous molecular complex suspension, α-CD. However, the biological half-life ($t_{1/2}$) was the same in both routes, approximately 20 hours. Absorption of CTFT from the α-CD vehicle was found to be much faster than from corn oil. The average $t_{1/2}$ of the absorption phase for a 10 mg/kg dose of CTFT in the α-CD and corn oil vehicles was 7 and 150 minutes, respectively. Despite the differences in absorption, no statistical difference was observed

in the calculated area under blood concentration versus time curves (AUC) obtained from rats dosed with CTFT in either vehicle. Blood concentrations of CTFT were proportional to dose, at levels as high as 400 mg/kg in both vehicles. The bioavailability of CTFT was shown to be complete in both vehicles, through comparing the AUC following oral and i.v. dosing.

In 14-day toxicity studies, 1 of 10 female rats given the top dose of 1000 mg/kg CTFT in corn oil died on day 8; no deaths of male rats or of mice of either sex were attributable to the administration of CTFT. Body weight gains in all groups of rats and mice were similar with the exception of the top dose (1000 mg/kg) groups of male and female rats, which lost weight during the first week and resumed weight gain during the second.

CTFT was found to accumulate in the kidneys of male rats, and there was a linear relationship between the kidney CTFT concentrations and the kidney levels of α2u-globulin, as determined by an ELISA assay. Microscopic changes in male rats included a dose-related toxic nephropathy consistent with that previously described as "hyaline droplet nephropathy." Dosed male and female rats also had hepatocyte hypertrophy and cytoplasmic vacuolization of the adrenal cortex. Clinical pathology findings suggested a mild anemia and cholestasis in rats. In contrast to rats, mice did not show appreciable CTFT concentrations in any tissue evaluated, suggesting a more rapid elimination of the chemical. However, hepatocellular hypertrophy, and clinical pathology findings consistent with cholestasis and mild liver injury, were noted in mice in the 400 and 1000 mg/kg dose groups.

These studies demonstrated that oral doses of CTFT of 400 mg/kg or higher caused liver hypertrophy in rats and mice and adrenal changes in rats. Doses of 50 mg/kg or higher caused "hyaline droplet nephropathy" in male rats. The results were similar with CTFT administered either in corn oil or in α-CD (although absorption of CTFT was somewhat more rapid with α-CD), suggesting that α-CD may be an appropriate vehicle for toxicity studies with other chemicals.

PEER REVIEW PANEL

Members of the Technical Reports Review Subcommittee of the National Toxicology Program's Board of Scientific Counselors who evaluated the draft report on the toxicity studies on *p*-chloro-α,α,α-trifluorotoluene on March 11-12, 1991, are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, panel members act to determine if the design and conditions of the NTP studies were appropriate and to ensure that the toxicity study report presents the experimental results and conclusions fully and clearly.

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Summary of Peer Review Comments

Dr. C.W. Jameson, NIEHS, NTP staff scientist, introduced the short-term toxicity studies of *p*-chloro-α,α,α-trifluorotoluene (CTFT) by reviewing the study rationale, experimental design, and results.

Dr. Klaassen, a principal reviewer of the draft study, stated that it was a good report and said he welcomed the inclusion of the pharmacokinetic data. He suggested that the NTP consider measuring levels of cytochrome P-450 isozymes, especially with chemicals that increase liver weight as was the case here. Dr. Jameson agreed that such measurements would be desirable, especially for chemicals for which liver is the target organ.

Dr. Goodman, the second principal reviewer, said that the study title should indicate that 2 dose vehicles (α-cyclodextrin and corn oil) were used, since this was a unique feature of the experiment. He also said the term "molecular encapsulation" needed better explanation, and questioned whether the α-cyclodextrin was preferable to corn oil as a gavage vehicle.

Dr. Jameson said the term “molecular complexation” might be a more appropriate term than “encapsulation.” A description of molecular complexation had been included in an earlier draft of the report but was deleted later; it would be included in the final report. Dr. Jameson also noted that the presence of CTFT in ground water made a drinking-water study desirable, but that limited solubility of CTFT in water was a problem. The more rapid absorption of CTFT from the α-cyclodextrin, as opposed to corn oil, was felt to better mimic the absorption from water.

Dr. Goodman and Dr. Zeise questioned the adequacy of the biexponential equation described in the report as giving the best fit to the data presented on the elimination of CTFT from blood following oral administration. Dr. Yuan replied that he believed the rather poor fit, primarily with the corn oil vehicle, was a reflection of the prolonged absorption phase, and that the biexponential equation, while not able to account for this effect, still provided the best fit of all models tried.

Following a short discussion of other editorial matters, Dr. Longnecker accepted the report on behalf of the panel, with the indicated revisions.

INTRODUCTION

Physical Properties, Production, Uses, and Exposure

p-Chloro-α,α,α-trifluorotoluene (CTFT) is a volatile, colorless, aromatic liquid with a melting point of -36°C, a boiling point of 139.3°C, a vapor pressure of 10 mm Hg at 29.5°C, and density of 1.353. It is sparingly soluble in water (≤ 1 mg/ml at 20°C) but is soluble in most organic solvents (Hawley, 1977).

CTFT is synthesized from the reaction of 4-chlorotoluene and anhydrous hydrogen fluoride under atmospheric or high pressure conditions (Grayson, 1980). U.S. production of CTFT in 1977 was estimated to be between 4.3 and 23 million kg (USEPA, 1985). Current production figures are not available. CTFT is mainly used as a chemical intermediate in the manufacture of dinitroaniline herbicides such as Trifuralin® (Carere *et al.,* 1981) and also is used as a dye intermediate and solvent and as a dielectric fluid (Hawley, 1977).

In 1977, CTFT was identified as one of 3 main pollutants in an environmental contamination accident near Vicenza in northeastern Italy; the CTFT concentration ranged from 0.05 to 90 µg/L in ground water (Belsito *et al.,* 1979). CTFT has been detected in water samples from Lakes Erie and Ontario and the Niagara river, (El-Saarawi *et al.,* 1985) and, along with other halogenated compounds, in Niagara river fish (Yurawecz *et al.,* 1979).

Absorption, Metabolism, and Excretion

No data were available on human absorption, metabolism, or excretion of CTFT. Metabolic products of CTFT in adult Sprague-Dawley rats, given a single oral 1 mg/kg dose of ^{14}C-labeled CTFT in corn oil, included glucuronides of dihydroxybenzotrifluoride and 4-chloro-3-hydroxybenzotrifluoride (each representing 3 - 4% of the administered ^{14}C). Minor amounts of a mercapturic acid conjugate of CTFT also were found among such products. CTFT was expired by rats (62 - 82% of the administered dose in 24 hours) and was the major ^{14}C-labeled residue in feces. Levels of ^{14}C-labeled residues in tissues were low, but the small amount of radiolabel in the rat carcass 4 days after dosage, approximately 1% of the applied dose, was identified as CTFT and was located mainly in fat (Quistad and Mulholland, 1983).

Toxic Effects

CTFT has a low acute oral toxicity, with an oral LD_{50} reported to be 13000 mg/kg in rats and 11500 mg/kg in mice (RTECS, 1988).

The toxicity of CTFT was assessed in Sprague-Dawley rats treated daily for 28 days by gavage with 0, 10, 100, or 1000 mg CTFT/kg (Macri *et al.,* 1987). No clinical signs other than salivation were observed in the high-dose group. Male rats showed a decrease in body weight gain but no concurrent decrease in food consumption. There were dose-dependent increases in blood cholesterol and triglycerides in males, suggesting alterations in lipid metabolism. Female rats showed a small dose-related increase in serum lactate dehydrogenase. Specific histological

alterations were found in the males in the 1000 mg/kg group, namely hyaline droplet nephrosis; they also displayed an increase in relative kidney weight and in lipid vacuoles in the adrenal cortex. Male and female rats in the 1000 mg/kg group showed an increase in relative liver weights. Slight nephrosis was observed in males given 100 mg/kg/day, but no pathological or biochemical alterations were found in rats given 10 mg/kg/day.

Genetic Toxicity

CTFT was tested, with concentrations up to 1000 μg/plate, in *Salmonella typhimurium* strains TA100, TA1535, TA1537, and TA98, in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver S9. No induction of gene mutations was observed in any of the strains (Haworth *et al.*, 1983).

In addition, Bignami and Crebelli (1979) and Benigni *et al.* (1982) reported negative results in *Salmonella* gene mutation tests with CTFT. Additionally, Benigni *et al.* (1982) tested CTFT for induction of mitotic recombination in *Aspergillus nidulans* and for unscheduled DNA synthesis (UDS) in human embryo epithelial cell cultures. The chemical induced UDS at concentrations of 1.0, 2.0, and 10.0 μl/ml, but no increase in mitotic recombination in *Aspergillus* was observed.

Environmental Toxicity

CTFT is phytotoxic towards corn root and potato disk cultures (Vianello *et al.,* 1981). It inhibits root growth by disrupting the uptake of sulfate. In contrast, Trifluralin® inhibits plant growth by disrupting both nuclear and cell division and the uptake of essential nutrients.

Rationale for Conducting Studies

The National Cancer Institute nominated CTFT for study, based on the potential for human exposure as indicated by its 1977 U.S. production range. Interest in CTFT also stemmed from the suspicion that it may be a carcinogen, because of structural similarities to *p*-chloro-α,α,α-trichlorotoluene, a carcinogen in mice (NCI/SRI, 1981), and to Trifluralin®, which also is carcinogenic in rodents (NCI, 1978). Study of CTFT also allowed the possibility for evaluating the toxic effect of the α,α,α-trifluoro moiety. CTFT is listed as a priority chemical under the Toxic Substances Control Act (USEPA, 1982) and was among 38 chemicals, from a list of 359, found in hazardous waste sites and judged to have a high potential for human exposure based on U.S. production and import data.

Oral administration was chosen for these studies because CTFT has been detected in ground water. The gavage method was selected, with corn oil as a vehicle, due to the volatility (b.p. 139°C) and low solubility of CTFT in water. Because increased incidences of pancreatic lesions have been observed in some longer-term studies in male F344/N rats administered corn oil by gavage (Boorman and Eustis, 1984; Eustis and Boorman, 1985; Haseman *et al.* 1985), an alternative gavage vehicle was evaluated as part of this study, using a molecular complex technique. α-Cyclodextrin (α-CD) was used as molecular *complexer,* with CTFT as a model compound. Comparative studies of the toxicity and toxicokinetics of CTFT were performed with the different gavage vehicles.

MATERIALS AND METHODS

Procurement and Preparation of CTFT with α-Cyclodextrin and with Corn Oil

CTFT (Lot # LV01811LM) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Analysis of the chemical by Midwest Research Institute (Kansas City, MO) indicated purity of > 97 %. α-Cyclo-dextrin was purchased from American Maize Products Co. (Hammond, IN).

CTFT (8 ml) was added to a 13.6% (w/v) α-CD aqueous solution to yield a concentration of 21.4 mg CTFT/ml. A white precipitate of molecularly complexed CTFT was formed. The mixture was stirred continuously for 3 days, then stored under refrigeration to accelerate precipitation and to facilitate the formation of fine particles. Although the suspension settled out during storage, it could be resuspended rapidly and homogeneously by stirring. Other suspensions, in concentrations as high as 80 mg CTFT/ml, could be prepared similarly.

Since CTFT is miscible with corn oil, the required amount of CTFT was added directly to corn oil (Mazola, CPC® International) for preparations with this vehicle. Both CTFT-α-CD and CTFT-corn oil preparations were analyzed by the gas chromatography method described below.

Dose Formulation Analysis Method

The CTFT/α-CD formulations were resuspended and an aliquot of the suspension (1 - 5 ml) was mixed with 5 ml of 0.5 N NaOH to release CTFT from the α-CD. Hexane (10 ml) was added and vortexed 4 minutes to extract the CTFT from the aqueous layer. An aliquot of the hexane extract was mixed with an equal volume of a tridecane internal standard solution (0.4 mg/ml), then injected directly into a Perkin-Elmer Sigma 2000 gas chromatograph (Perkin-Elmer Corp., Norwalk, CT), equipped with a flame ionization detector. The injector and detector temperatures were 200 and 300°C, respectively. Oven temperatures were programmed from an initial 65°C with a hold time of 2.5 minutes, to 125°C, increasing at a rate of 10°C/min. The column (2 mm x 1.8 m) was packed with 10% SP-1000 on 80/100 Supelcoport® (Supelco, Inc., Bellafonte, PA); the nitrogen carrier gas had a flow rate of 30 ml/min.

In preparing standards, equal amounts of CTFT were added to equal volumes of hexane and of tridecane internal standard solution. Plotting the peak area ratio of CTFT and tridecane against CTFT concentrations in hexane yielded the standard curve. Concentrations of CTFT were calculated from the standard curve after correcting for sample size and dilution.

For the CTFT-corn oil formulations, aliquots (0.1 ml) were diluted with hexane (10 ml). The hexane solution (1 ml) then was mixed with an equal volume of tridecane internal standard solution (0.4 mg/ml) and analyzed by gas chromatography.

14-Day Toxicity Study

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Charles River Breeding Laboratories (Wilmington, MA) and were quarantined and acclimated to laboratory conditions for

14 days before study start. Rats were housed 3 per cage, and mice 5 per cage, on hardwood chip bedding in polystyrene cages covered with polyester filter sheets. The animal room was maintained at 22 ± 2°C, and 50 ± 10% relative humidity, with an artificial 12-hour light/dark cycle. Animals were allowed access to food (NIH-07, Zeigler Bros., Gardners, PA) and water *ad libitum.*

Groups of 5 animals per sex were administered either CTFT-α-CD suspension or CTFT-corn oil solution by gavage (5 ml/kg for rats, 10 ml/kg for mice) between 9:00 and 11:00 a.m. daily for 14 consecutive days. Additional groups of 5 male rats each were administered CTFT at all dose levels and used exclusively for the determination of α2u-globulin in the kidney. Rats and mice received 0, 10, 50, and 400 mg CTFT/kg in CTFT-α-CD suspensions, or 0, 10, 50, 400, and 1000 mg CTFT/kg (mice) and 0, 50, 400, and 1000 mg CTFT/kg (rats) with the corn oil vehicle. Dose levels were based on the results of the CTFT toxicity study performed with rats, reported by Macri *et al.* (1987), and on the maximum loading capacity of the α-CD vehicle.

Body weights were measured every other day and at necropsy. Twenty-four hours after the last dosing, blood samples were drawn from the orbital sinuses of rats and mice, prior to termination by CO_2 anesthesia, for clinical chemistry and hematology evaluation and for analysis of blood CTFT concentrations. Tissue samples also were taken from the liver and kidney for analysis of CTFT. Complete necropsy examinations were performed on all rats and mice. Organ weights were determined to the nearest milligram for the liver, right kidney, heart, and lungs, and to the nearest 0.1 milligram for the thymus and right testis. All tissues were fixed in formalin; the tissues listed in Table 1 were processed, sectioned, and stained with hematoxylin and eosin for microscopic examination. The study pathologist examined all organs from high dose and control animals of each sex and species. All gross lesions and target organs were examined in all other dose groups (Table 1). Each of these slides was reviewed by a second pathologist; in instances where the study pathologist and reviewing pathologist disagreed, the final diagnosis represents results of a reexamination of the slides and consensus between the two pathologists. Additional sections of kidney from all male rats were stained by the Mallory-Heidenhain method to evaluate the morphology of the protein droplets ("hyaline droplets") in the tubular epithelium and lumen. Samples of liver and kidney were fixed in Fowler's solution (2.5% glutaraldehyde/2.0% formaldehyde) for electron microscopy. Ultrastructural examination of liver, kidney, and adrenal gland was performed on samples from selected high-dose rats and control male and female rats.

Toxicokinetic Studies

Adult male F344/N rats (290-310 g) were obtained from Charles River Breeding Laboratories (Raleigh, NC), then quarantined for 2 weeks. The animals were housed in polystyrene cages in a room maintained at 22 ± 2°C and 50 ± 10% relative humidity with an artificial 12-hour light/dark cycle. Rats were allowed access to food (NIH-07, Zeigler Bros., Gardners, PA) and water *ad libitum* prior to treatment. Three animals per dose per vehicle were used. Rats were anesthetized with a Ketamine® HCl (100 mg/ml) and Xylazine® HCl (3.3 mg/ml) mixture at a dose of 0.1 ml/100 g body weight. Then the jugular vein was cannulated with a 0.020 in. (i.d.) by 0.037 in. (o.d.) Silastic® medical grade tubing (Dow Corning Corporation, Midland, MI) and the rats were allowed

TABLE 1 Experimental Design and Materials and Methods in the 14-Day Gavage Studies of *p*-Chloro-α, α, α-trifluorotoluene

Study Date
February - March, 1990

Animal Source
Charles River Breeding Laboratories, Wilmington, MA

Chemical Source
CTFT: Aldrich Chemical Company, Milwaukee, WI
α-Cyclodextrin (α-CD): American Maize Products Company, Hammond, IN

Size of Study Groups
5/sex/group of each species; rats housed 3 per cage; mice housed 5 per cage.

Doses
Rats--Gavage
Corn oil: 0, 50, 400, and 1000 mg/kg
α-CD: 0, 10, 50, and 400 mg/kg

Mice--Gavage
Corn oil: 0, 10, 50, 400, and 1000 mg/kg
α-CD: 0, 10, 50, and 400 mg/kg

Study Laboratory
NIEHS, Research Triangle Park, NC;

Strain and Species
F344/N rats; B6C3F$_1$ mice

Method of Animal Distribution
Weight-randomized

Diet
NIH 07; available *ad libitum*

Animal Room Environment
Temperature--20-24°C; relative humidity--50±10%; fluorescent light 12 hours/day; 10 room air changes/hour.

Time Held Before Study
2 weeks

Age When Placed on Study
10-14 wks

Age When Killed
12-16 wks

Type and Frequency of Observation
Observed 2x day, weighed every other day and at terminal sacrifice.

Necropsy and Histologic Examinations
Necropsy performed; tissues preserved in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with H&E for microscopic examination. The following tissues were examined microscopically from all high-dose and control animals and all early deaths in other groups: adrenal gland, bone and bone marrow, brain, cecum, clitoral/ preputial glands, colon, duodenum, mesenteric lymph node, epididymis, esophagus, gall bladder (mice), heart, ileum, jejunum, kidney, lung, liver, mammary gland, mandibular lymph node, nasal cavity, ovary, pancreas, pituitary gland, prostate, parathyroid gland, rectum, salivary gland, skin, spleen, stomach, seminal vesicle, testis, thyroid, thymus, trachea, urinary bladder, uterus, and all gross lesions. In addition to gross lesions, the following tissues were examined in all other dosed groups: liver, kidney, adrenal gland (rats) and liver (mice). Hematologic and serum chemical analyses were performed; residues of CTFT were measured in the blood, kidney, and liver of rats and mice at study termination.

to recover for at least 24 hours. Body weights were measured and the cannula flushed with heparin-saline solution before dosing.

Three rats per dose group were given a CTFT-α-CD formulation by gavage at levels of 10, 50, 200, or 400 mg/kg body weight, or a CTFT-corn oil formulation at levels of 10, 50 or 400 mg/kg body weight. A dosing volume of 5 ml/kg body weight was used. At predetermined intervals, 200 µl blood samples were drawn via the cannula and dispensed into pre-weighed, capped glass vials containing 2 mg EDTA and 1 ml of a solution of internal standard in hexane (1 µl 1,3-dichlorobenzene in 1000 ml hexane). Approximately 18 blood samples were collected over a period of about 52 hours; each vial was weighed to obtain the amount of blood sampled. The vials were stored at –10°C for no longer than 2 weeks before analysis.

In the i.v. study, 2 cannulated rats were administered 10 mg/ml CTFT dissolved in 10% Tween 80 aqueous solution at 4.7 mg/kg body weight, via the tail vein. Blood samples taken at predetermined time intervals were handled as described above; all were analyzed by a GC method with ECD detector. The GC injector, ECD detector, and oven temperatures were 230, 390, and 100°C, respectively. The column used was a 10% SP-1000 on 80/100 Supelcoport® (2 mm x 1.8 m) with an argon/10% methane carrier gas at a flow rate of 30 ml/min.

Method of Analysis of CTFT Concentrations in Blood, Kidney, and Liver

Blood/hexane samples obtained above were agitated in a mechanical shaker for 45 minutes and centrifuged for 3 minutes at 1500 rpm. The hexane phase then was transferred to GC auto-sampler vials. Standards were prepared by adding CTFT-methanol stock solutions (5 - 20 μl) into aliquots of control rat blood (200 μl). The resulting standards were analyzed with each group of blood samples. Three linear standard curves (0.04 - 0.2 μg/ml, r = 0.996, 0.2 - 5.0 μg/ml, r = 0.998 and 5.0 - 50 μg/ml, r = 0.999) covering the expected blood concentrations were constructed by plotting peak area ratios of CTFT and internal standard versus concentrations. CTFT concentrations in rat blood samples were calculated from the corresponding standard curves. The method was evaluated over the 3 concentration ranges. The relative standard deviation in all cases was < 10 %; the quantitation limit was 0.04 μg/ml of blood. CTFT was found to be stable in control rat blood stored at -10°C for at least 2 weeks.

The entire left kidney and a portion of the liver from rats and mice were weighed, then homogenized separately with ice water (8 - 15 times sample weight), using a Brinkmann PolyTron® (Brinkmann Instruments, Inc., Westbury, NY). An aliquot of the homogenate was transferred to a capped vial; hexane, containing internal standard (3 μl 1,3-dichlorobenzenze in 1000 ml hexane), was added to extract the CTFT. The hexane extract then was analyzed with the gas chromatographic method described above. Linear standard curves were constructed by spiking control tissue homogenate with a methanolic solution of CTFT. CTFT concentrations in rat and mice tissue samples were calculated from standard curves constructed as outlined above. The method was evaluated with spiked samples of both kidney and liver. The concentration range validated for liver was 0.1 - 1.0 μg per 250 μl of homogenate solution (r = 0.999); for kidney, the concentration ranges were 0.2 - 1.2 and 1.2 - 14 μg per 250 μl of homogenate (r = 0.999 and 0.997, respectively). The relative standard deviation in all cases was < 9 %. The quantitation limit was 0.1 μg per 200 μl tissue homogenate (or 3.6 μg/g tissue). Recovery of CTFT from kidney and liver homogenates ranged from 90 to 100%.

Kidney Total Protein and α2u-Globulin Determination

The entire right kidney of male rats was homogenized with 2 times its volume of phosphate-buffered saline (pH 7.2) at 4°C, then stored at -20°C until analysis. Kidney homogenates were thawed, then centrifuged at 2000 RPM for 10 min. Total protein content in the supernatant was determined by the bicinchoninic acid assay (Kit No. BCA-1, Sigma Chemical Co., St. Louis, MO; Smith *et al.,* 1985). The amount of α2u-globulin in the supernatant was determined by an ELISA assay, as described by Charbonneau *et al.* (1987). The standard α2u-globulin and the antibody (a mouse immunoglobulin G raised toward rat α2u-globulin) for ELISA were provided by Dr. S. Borghoff (Chemical Industry Institute of Toxicology, Research Triangle Park, NC). The second antibody (anti-mouse IgG) conjugated with alkaline phosphatase was obtained from Sigma Chemical Co. (St. Louis, MO). Results were expressed as the ratio of α2u-globulin to total protein in the supernatant.

Clinical Pathology

Rats and mice were anesthetized with a mixture of CO_2 and O_2 (70%:30%); blood samples were collected from the retroorbital sinus using heparinized microcapillary tubes. In rats, samples for determination of hematologic and biochemical variables were collected from the same animals, in the same sequence (i.e., sampled for hematology before that for biochemistry). Mice were sampled only for biochemical analyses. Blood samples for hematologic analyses (approximately 0.50 ml) were collected in plastic tubes coated with potassium EDTA (Microvette CB 1000, Sarstedt, Numbrecht, Germany) and held at room temperature. Samples for biochemical analyses (approximately 0.75 ml) were collected in plastic serum separator tubes containing serum separator gel (Microtainer, Becton Dickinson, Rutherford, NJ). These samples were allowed to clot for 30 minutes at room temperature, then were centrifuged at 5000 g for 10 minutes. The serum then was removed for biochemical analyses.

Hematologic and Biochemical Analyses

Automated hematologic analyses were performed with an H-1 hematology system (Technicon Instruments Corp., Tarrytown, NY). The following variables were measured using software developed for rodents: erythrocyte, leukocyte, and platelet counts; mean corpuscular volume (MCV); mean corpuscular hemoglobin concentration (MCHC); mean corpuscular hemoglobin (MCH); hematocrit (HCT); and hemoglobin concentration (HGB). Leukocyte differentials were determined by microscopic evaluation of blood smears stained with Wright-Giemsa. Reticulocyte counts were determined using smears of preparations containing equal volumes of whole blood and new methylene blue stain, incubated at room temperature for 20 minutes.

Biochemical analyses of serum were performed using a Monarch 2000 chemistry system (Instrumentation Laboratories, Lexington, MA). Reagent kits and applications developed by the manufacturer were used for the following variables: alanine aminotransferase (ALT), total protein, albumin, glucose, triglycerides, cholesterol, urea nitrogen (UN), creatinine, creatine kinase (CK), and alkaline phosphatase (AP). Determinations of 5'-nucleotidase, sorbitol dehydrogenase (SDH), and total bile acids (TBA) were obtained using reagent kits from Sigma Chemical Co. (St. Louis, MO); applications were developed in-house for the chemistry analyzer.

Toxicokinetic Analysis

Average and individual blood CTFT concentrations, relative to the time after intragastric or i.v. dosing for each dose group, were evaluated for agreement with several toxicokinetic models using the program NONLIN® (Metzler *et al.*, 1974). Using the same program, CTFT absorption rates were estimated individually for each rat. Preliminary values used in the NONLIN® program were obtained by curve-stripping using the program, JANA® (Statistical Consultants Inc., Lexington, KY). The terminal elimination rate was determined by plotting blood concentration versus time, and a linear regression analysis of the terminal data points. Model independent parameters were calculated as follows:

The AUC parameter was defined as:

$$AUC = \int_0^{\infty} C(t)\,dt \qquad (1)$$

and was calculated by the trapezoidal rule with end point correction:

$$AUC = \sum_{i=0}^{i=n} 1/2\,(C_{i+1} + C_i)(t_{i+1} - t_i) + \frac{C_{n+1}}{\gamma} \qquad (2)$$

where C_i is the blood concentration at time t_i; γ is the terminal elimination rate constant, and C_{n+1} is the last blood concentration point measured.

The total body clearance(CL_T) of CTFT was calculated using the formula:

$$CL_T = F(Dose)/AUC \qquad (3)$$

where F is the bioavailability (in the i.v. route, F=1). Results of CL_T were then converted to ml/min/kg units for convenience in presenting the data.

Apparent volume of distribution (V_d) was calculated using the formula:

$$V_d = \frac{Dose}{\gamma * AUC} \qquad (4)$$

Results of V_d were then converted to L/kg again for convenience.

The biological half-life ($t_{1/2\gamma}$) of CTFT in blood was calculated by the formula:

$$t_{1/2\gamma} = 0.693/\gamma \qquad (5)$$

The absorption half-life ($t_{1/2k_a}$) of CTFT was calculated by the similar formula:

$$t_{1/2k_a} = 0.693/k_a \qquad (6)$$

The bioavailability of orally administered CTFT was determined using the formula:

$$\text{Bioavailability} = [(AUC)_{po}/Dose_{po}] \Big/ [(AUC)_{iv}/Dose_{iv}] \qquad (7)$$

Statistical Methods

Changes in toxicokinetic parameters were evaluated for statistical significance by 2-way ANOVA as a function of dose, vehicle, and the interaction of dose and vehicles using the program Data Desk®

(Odesta Corporation, Northbrook, IL), with a significance threshold at $p < 0.01$. The linear regression analysis of AUC versus dose was performed using the same program.

Body- and organ-weight changes in animals were evaluated by 2-way ANOVA as a function of dose and vehicle. Dose and vehicle interactions also were evaluated, using the Student *t*-test.

The significance of differences between dosed and control groups for clinical chemistry and hematology endpoints was assessed using nonparametric multiple comparison procedures for protection against false-positive inferences. Either Dunn's test or Williams' modification of Shirley's multiple comparisons procedures was applied, based on the occurrence of a dose-related response in the data (Dunn, 1964; Shirley, 1977; Williams, 1986). If the p-value from Jonckheere's test (Hollander and Wolfe, 1973) for a dose-related trend was ≥ 0.1, Dunn's test was used instead of Shirley's test. Tables for individual parameters show the results of Dunn's or Shirley's test.

RESULTS

Toxicokinetics of CTFT Using α-CD and Corn Oil Vehicles: I.V. Study

Two male rats were administered an aqueous CTFT-Tween 80 formulation (4.7 mg/kg) via the tail vein. Serial blood samples were collected from each rat via jugular vein cannula and analyzed by gas chromatography.

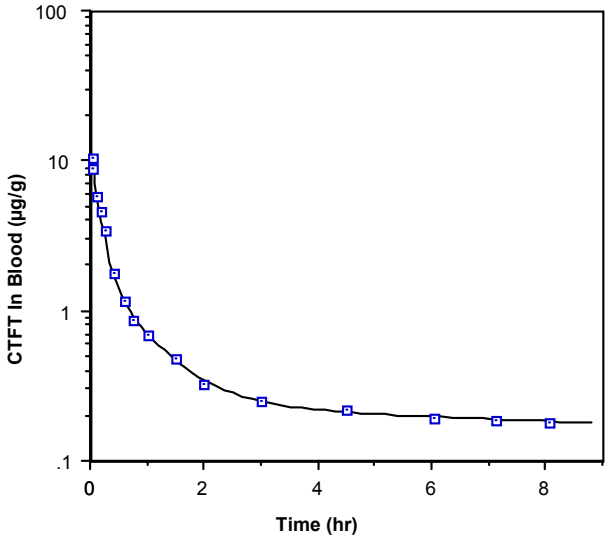

Figure 1

Average CTFT blood concentration vs. time profile of 2 male F344/N rats after intravenous administration of aqueous 10% Tween 80, CTFT solution at 4.7 mg/kg by tail vein injection. The solid curve is the best fit using a 3- compartment model: $C = 7.63\ e^{-30.5t} + 6.88\ e^{-3.55t} + 0.26\ e^{-0.036t}$

The blood CTFT concentration (log scale) versus time data obtained from the i.v. studies were best fit by the following triexponential equation:

$$C_p = A\ e^{-\alpha t} + B\ e^{-\beta t} + C\ e^{-\gamma t}$$

where C_p is the blood concentration of CTFT; α, β, and γ are rate constants for 2 distribution and 1 elimination phase; and A, B and C are the zero-time intercepts of the 3 exponential phases, respectively. Figure 1 shows the average blood concentration versus time profile for 2 rats that received a 4.7 mg/kg i.v. dose of CTFT, and the 3-compartment modeling results (solid line). The 3-compartment model would appear to describe the data adequately. The biological half-life ($t_{1/2\gamma}$) of CTFT in blood was approximately 19 hours. The elimination rate constant γ (0.036 hr^{-1}), total body clearance CL_T (7.8 ml/min/kg), area under curve AUC (9.9 µg/ml • hr), dose normalized AUC (AUC/Dose, 2.1 µg•kg/ml•hr•mg), and apparent volume of distribution V_d (13.1 L/kg) were calculated using the equations described in the Materials and Methods section.

Toxicokinetics of CTFT Using α-CD and Corn Oil Vehicles: Gavage Study

CTFT was administered by gavage in either an α-CD or corn oil vehicle, at doses of 10, 50, or 400 mg/kg. The decline of CTFT concentrations in blood was best described by the equation:

$$Cp = B\ e^{-\beta t} + C\ e^{-\gamma t} - (B+C)\ e^{-k_a t}$$

where k_a was the absorption rate constant, dependent upon both the rate of release of the chemical from the vehicle and the rate of the absorption of the chemical from the gastrointestinal tract. The α distribution phase was masked totally by the absorption phase, particularly with the corn oil vehicle. These results indicate that the rate of distribution (α) is much faster than the rate of absorption. The average blood concentration versus time profiles are shown in Figure 2 and the modeling results are presented in Table 2. A linear scale, rather than log scale, is used for the Y axis in Figure 2, to illustrate more clearly the different absorption characteristics of the corn oil and α-CD vehicles. The time span shown in Figure 2 is much larger than Figure 1; the first few data points in Figure 2 are compressed, compared to those in Figure 1.

TABLE 2 2-Compartment Pharmacokinetic Analysis Results

Vehicle	Dose (mg/kg)	Modeling Equation[a] $C_p = B\ e^{-\beta t} + C\ e^{-\gamma t} - (B+C)\ e^{-k_a t}$		
α-CD	10	$C_p = 33.6\ e^{-2.86t}$	$+ 1.00\ e^{-0.038t}$	$- 34.7\ e^{-3.31t}$
	50	$C_p = 2.88\ e^{-0.57t}$	$+ 4.07\ e^{-0.035t}$	$- 6.96\ e^{-5.78t}$
	400[b]	$C_p = 16.5\ e^{-0.17t}$	$+ 30.8\ e^{-0.034t}$	$- 47.3\ e^{-2.39t}$
Corn oil	10	$C_p = 1.93\ e^{-0.084t}$	$+ 0.076\ e^{-0.040t}$	$- 2.00\ e^{-0.89t}$
	50	$C_p = 3.99\ e^{-0.09t}$	$+ 3.36\ e^{-0.034t}$	$- 7.35\ e^{-0.49t}$
	400	$C_p = 110\ e^{-0.16t}$	$+ 28.3\ e^{-0.035t}$	$- 134\ e^{-0.26t}$

a Obtained by curve fitting the average data from 3 rats for each dose.
b Obtained by curve fitting the average data from 2 rats.

A number of toxicokinetic parameters calculated from the above data are presented in Table 3. Two-way ANOVA demonstrated that there were no significant differences between the α-CD and corn oil vehicles for the terminal elimination rate constant (γ), total body clearance (CL_T), area under the curve (AUC), dose-normalized AUC (AUC/dose), and apparent volume of distribution (V_d).

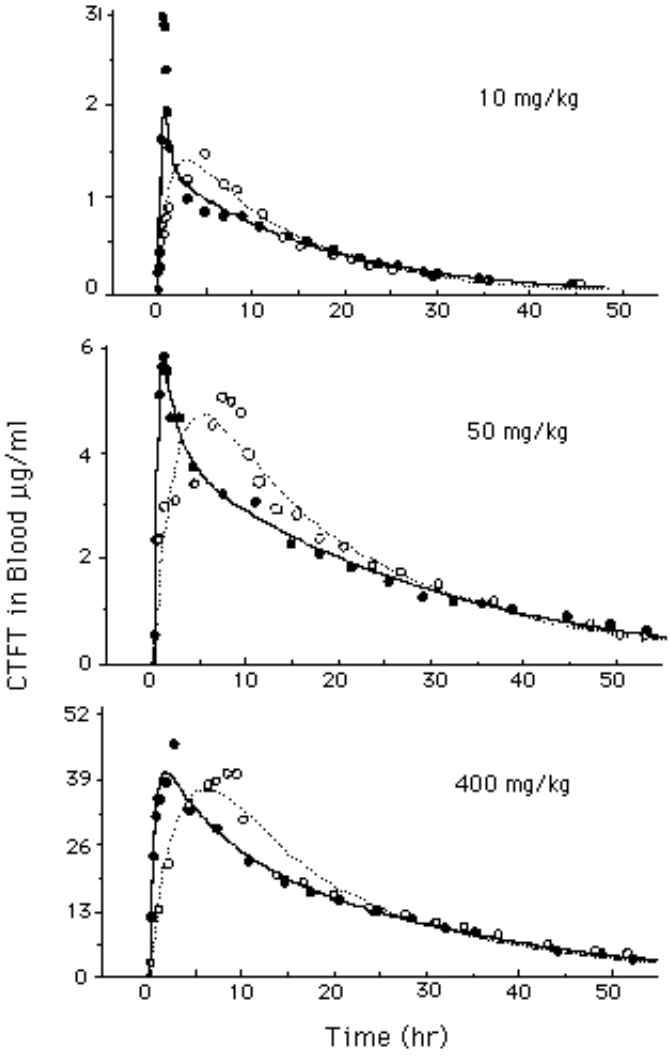

Figure 2

Average CTFT blood concentrations of F344/N male rats after gavage administration of CTFT with α-CD vehicle (•) or corn oil vehicle (◦). Each point is the average value of 3 rats, except at 400 mg/kg with the α-CD vehicle, where 2 rats were used. The solid (α-CD) and dashed (corn oil) curves are the best fits using a 2-compartment model.

TABLE 3 Toxicokinetic Parameter Estimates[a] in Male F344/N Rats Following Gavage Administration of CTFT In α-CD or Corn Oil[b]

Vehicle	Dose (mg/kg)	Elimination rate constant γ (hr-1)	Volume of distribution Vd(L/kg)	Total Body Clearance CLT(mL/ min/kg)	T_{max} (hr)	C_{max} µg/mL	C_{max}/ Dose	Absorption Rate Constant K_a(1/hr)	AUC (µg/mL•hr)	AUC/Dose
α-CD	10	0.036±0.004	11.3±2	6.7±0.7	0.3±0.04	3.0±0.3	0.30±0.03	3.0±0.6	24.9±2.6	2.49±0.26
Corn Oil	10	0.038±0.006	11.1±2	6.8±0.3	6.8±1.7	1.4±0.2	0.14±0.01	0.9±0.4	24.4±1.0	2.44±0.10
α-CD	50	0.030±0.001	13.5±3	6.7±1.5	0.6±0.1	5.7±0.7	0.11±0.01	5.5±3	128.0±26	2.56±0.52
Corn Oil	50	0.033±0.002	11.6±1	6.4±1.3	7.7±0.6	5.4±0.8	0.10±0.02	0.5±0.2	130.0±3	2.60±0.06
α-CD	200[c]	0.028±0.008	16.5±4	7.6±0.4	1.8±0.6	23.0±4	0.12±0.02	3.2±2.8	437.0±20	2.18±0.10
α-CD	400[c]	0.033±0.004	12.0±3	6.4±0.8	1.6±1.2	52.4±11	0.13±0.02	2.0±1.6	1054.0±145	2.63±0.36
Corn Oil	400	0.034±0.001	11.6±2	6.6±0.9	8.3±1.4	41.8±8.7	0.10±0.02	0.3±0.3	1019.0±134	2.54±0.34

a Results are expressed as mean ± SD.
b n=3.
c n=2.

Significant differences ($p < 0.01$) in the toxicokinetic parameters between the 2 vehicles were found for time to reach the maximum blood concentration (T_{max}), maximum blood concentration achieved (C_{max}), dose normalized C_{max} (C_{max}/dose), and the absorption rate constant (k_a). In comparison to the corn oil vehicle, the α-CD vehicle formulation had a shorter T_{max}, higher C_{max} and C_{max}/dose and faster rate of absorption (Table 3). The calculated average absorption half-lives for the α-CD and corn oil vehicles were 17 and 98 minutes, respectively.

ANOVA revealed that the terminal elimination rate constant (γ), apparent volume of distribution (V_d), total body clearance (CL_T), time to reach the maximum blood concentration (T_{max}), absorption rate constant (k_a) and dose-normalized AUC (AUC/dose) are dose-independent over the range of 10 to 400 mg/kg. As expected, the maximum blood concentration achieved (C_{max}), dose normalized C_{max} (C_{max}/dose), and area under the curve (AUC) were found to be dose-dependent.

Linear regression analysis of the AUC versus dose showed that the AUC increased proportionally with dose for both vehicles (R^2= 0.979, $p < 0.01$). The plot of AUC data versus dose is shown in Figure 3.

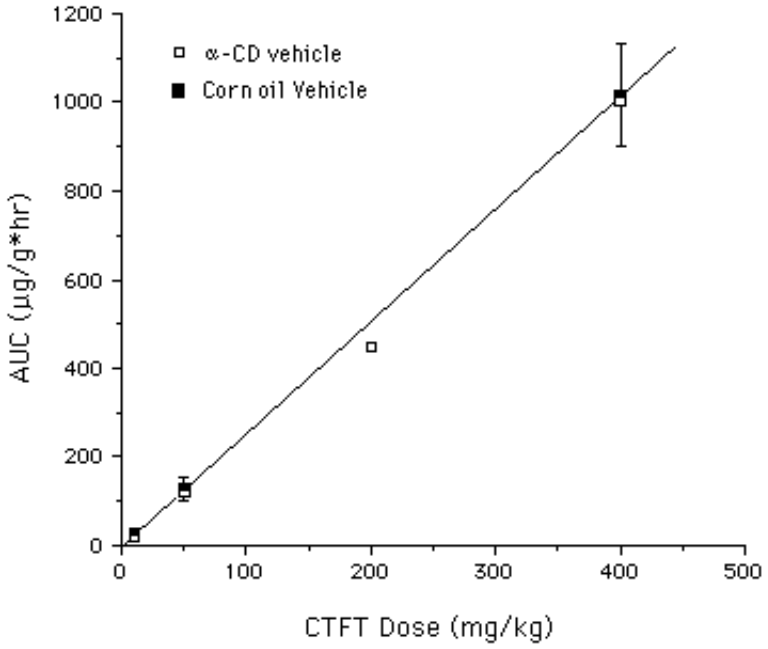

Figure 3
Dose proportionality of CTFT in male F344/N rats with α-CD vehicle and corn oil vehicle.
Regression analysis: AUC= 2.57 * Dose - 6, (R^2= 0.979, p<0.01).

The terminal elimination rate constant (γ), apparent volume of distribution (V_d), and total body clearance (CL_T) reflect the toxicokinetic behavior of CTFT. Because they are dose- and vehicle-independent as determined by ANOVA, individual values of these parameters can be combined to obtain the more accurate pooled value. The pooled values are: γ (0.034±0.004 1/hr); CL_T (6.7 ± 0.7 ml/min/kg); and V_d (11.9 ± 1.9 L/kg). These values are close to the values obtained from the i.v. study, confirming that these characteristic parameters for CTFT are independent of the route of administration.

Since the doses used in these studies were found to be within the dose proportional range, it was possible to calculate the absolute bioavailability of CTFT. The bioavailability from both vehicles was calculated to be greater than 100% (Table 4).

TABLE 4 Bioavailability of CTFT with α-CD Suspensions or Corn Oil Vehicle in Male F344/N Rats[a]

Route	Vehicle	AUC/Dose	Bioavailability(%)
I.V.[b]	10% Tween 80	2.1±0.1	100
Oral[c]	14% Aqueous α-CD	2.4±0.3	114±14
Oral[d]	Corn Oil	2.5±0.2	119±10

a Results are expressed as mean ± standard deviation; the AUC/dose values determined for the oral routes were significantly greater (p < 0.01, Student's t-test) than that determined for the i.v. route.
b n=2
c n=10
d n=9

14-Day Toxicity Study in F344/N Rats

Immediately following gavage dosing with CTFT, in either the α-CD or corn oil vehicle, the animals showed minimal adverse clinical signs (burrowing in bedding, rubbing face with forepaws) that were suggestive of chemical irritation. One female rat in the high-dose (1000 mg/kg) corn oil vehicle group died on day 8 of the study; the cause of death could not be determined. There were no gross lesions, and microscopic changes were limited to mild hepatocyte hypertrophy and fatty change in the liver.

There was no marked effect of CTFT administration on body weights, except among the 1000 mg/kg dose group using the corn oil vehicle. This group showed decreasing body weight during the first week in both males and females (Figure 4). The final body weight of males receiving 1000 mg/kg CTFT in corn oil by gavage was significantly lower than controls (Table 5).

TABLE 5 Survival and Weight Gain of F344/N Rats in the 14-day Gavage Studies of CTFT

Dose Level (mg/kg)	Survival[a]	Mean Body Weight (grams) Initial	Final	Change[b]	Final Weight Relative to Controls (%)[c]
Male					
Corn Oil Vehicle					
0	5/5	251	263	12	
50	5/5	256	263	7	100
400	5/5	255	257	2	98
1000	5/5	253	246	-7	94
α-CD Vehicle					
0	5/5	256	269	13	
10	5/5	254	263	9	98
50	5/5	255	267	12	99
400	5/5	254	265	11	99
Female					
Corn Oil Vehicle					
0	5/5	179	176	-3	
50	5/5	174	173	-1	98
400	5/5	178	177	-1	101
1000	4/5	181	172	-9	98
α-CD Vehicle					
0	5/5	181	172	-9	
10	5/5	177	170	-7	99
50	5/5	176	179	3	104
400	5/5	173	172	-1	100

a Number surviving at 2 weeks/number of animals per dose group.
b Mean weight change of the animals in each dose group.
c (Dosed group mean/Control group mean) x 100.

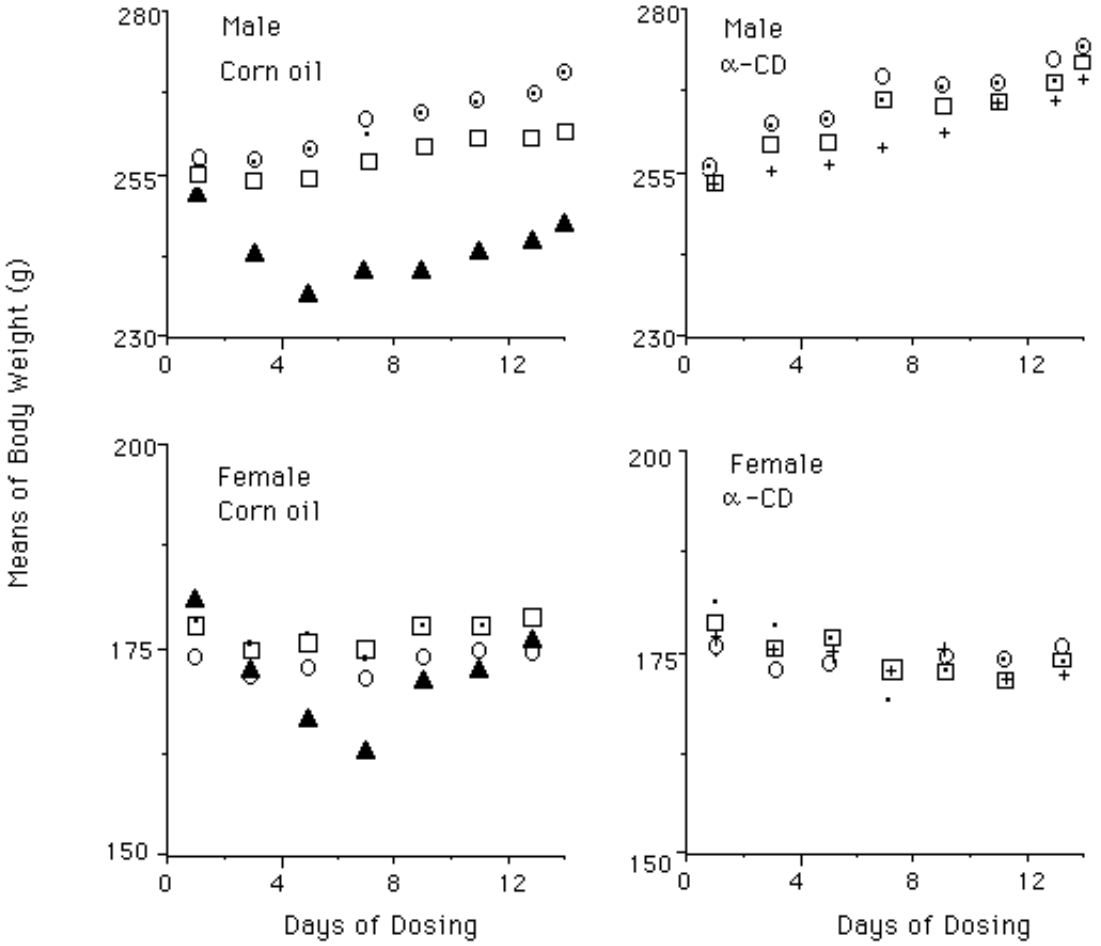

Figure 4
Mean body weights of male and female F344/N rats during the 14-day CTFT studies with corn oil and α-CD vehicles.
•Control group =10 mg/kg m50 mg/kg q400 mg/kg s1000 mg/kg

Organ weights of rats are presented in Tables 6 and 7. Dose-related increases in liver weights were observed in males at 50 mg/kg or higher and in females at 400 mg/kg or higher. Kidney weights were increased in males receiving CTFT at doses of 400 mg/kg and higher, and in females receiving 1000 mg/kg. The effects of CTFT on organ weights were similar with both vehicles (ANOVA results).

CTFT concentrations were determined in blood, kidney, and liver from all animals approximately 24 hours after the last gavage dose. The results are listed in Table 8. Three-way ANOVA showed that CTFT concentrations in blood, liver, and kidney were vehicle-independent (all $p > 0.22$) but were sex- and dose-dependent ($p < 0.001$).

The average concentration of CTFT in the blood of females given 1000 mg/kg CTFT in corn oil was higher than the corresponding value for the males. No differences in CTFT blood concentrations were noted between the sexes in other dose groups. A large difference was observed between kidney CTFT concentrations in males and females. The concentration of CTFT in the kidneys of males was about 10 times higher than in females and about 10 times higher than the blood

TABLE 6 Selected Organ Weights for Male F344/N Rats in the 14-Day Gavage Studies of CTFT[a]

Organ	Vehicle	Dose level of CTFT (mg/kg)[b] 0	10	50	400	1000
Necropsy Body Weight (g)	α-CD	269±6	263±9	267±8	265±7	–[c]
	corn oil	264±9	–[c]	263±7	257±6	246±5[e]
Organ Weight (g)						
Liver	α-CD	9.86±0.25	9.95±0.40	10.7±0.33[d]	13.7±0.66[e]	–
	corn oil	9.39±0.61	–	10.5±0.60[d]	13.3±1.3[e]	16.0±0.68[e]
Right Kidney	α-CD	0.97±0.08	1.12±0.34	0.98±0.02	1.16±0.07[d]	–
	corn oil	0.94±0.04	–	0.99±0.10	1.2±0.08[e]	1.14±0.05[e]
Heart	α-CD	0.99±0.04	0.99±0.09	1.04±0.07	1.00±0.07	–
	corn oil	0.96±0.04	–	0.96±0.04	0.99±0.09	0.93±0.06
Lung	α-CD	1.42±0.10	1.38±0.09	1.50±0.12	1.50±0.08	–
	corn oil	1.63±0.20	–	1.47±0.13	1.52±0.13	1.50±0.19
Right Testis	α-CD	1.46±0.03	1.46±0.05	1.42±0.06	1.53±0.06	–
	corn oil	1.43±0.07	–	1.47±0.08	1.46±0.08	1.48±0.05
Thymus	α-CD	0.35±0.06	0.36±0.02	0.37±0.05	0.33±0.05	–
	corn oil	0.39±0.05	–	0.43±0.09	0.31±0.07	0.32±0.06
Organ-to-Body-Weight Ratio (%)						
Liver	α-CD	3.7±0.08	3.9±0.21	4.0±0.12[d]	5.20±0.19[e]	–
	corn oil	3.6±0.16	–	4.0±0.19[d]	5.2±0.50[e]	6.5±0.21[e]
Right Kidney	α-CD	0.36±0.03	0.43±0.14	0.37±0.01	0.44±0.03[d]	–
	corn oil	0.36±0.01	–	0.38±0.04	0.47±0.03[e]	0.47±0.03[e]
Heart	α-CD	0.37±0.02	0.38±0.03	0.39±0.03	0.38±0.03	–
	corn oil	0.36±0.02	–	0.36±0.01	0.39±0.04	0.38±0.02
Lung	α-CD	0.53±0.04	0.53±0.05	0.56±0.05	0.57±0.03	–
	corn oil	0.62±0.08	–	0.56±0.05	0.59±0.06	0.60±0.07[d]
Right Testis	α-CD	0.54±0.01	0.56±0.03	0.53±0.03	0.58±0.03	–
	corn oil	0.54±0.03	–	0.56±0.03	0.57±0.04	0.60±0.02
Thymus	α-CD	0.13±0.02	0.14±0.01	0.14±0.02	0.12±0.02	–
	corn oil	0.15±0.02	–	0.16±0.033	0.12±0.02	0.13±0.02

a Mean ± standard deviation.
b n= 5 for all dose groups.
c No α-CD group at 1000 mg/kg or corn oil group at 10 mg/kg.
d Significantly different from 0 dose group value ($p < 0.01$).
e Significantly different from 0 dose group value ($p < 0.001$).

concentration, indicating a specific accumulation of CTFT in this organ in males. The concentration of CTFT in the liver of males also was higher than in females.

Using the data obtained from the toxicokinetic study of CTFT in male rats, described in Table 2, a theoretical CTFT blood concentration versus time profile during the 14-day study was simulated by computer for the 50 and 400 mg/kg dose groups, using a 2-compartment model with repeated dosing (Figure 5). The measured blood concentrations in both vehicle groups at both dose levels were very similar to those predicted by the model. Because no significant differences in CTFT blood concentrations were noted with the different vehicles, a similar toxicity response was expected.

TABLE 7 Selected Organ Weights for Female F344/N Rats in the 14-day Gavage Studies of CTFT[a]

Organ	Vehicle	Dose level of CTFT (mg/kg)[b] 0	10	50	400	1000
Necropsy Body Weight (g)	α-CD	172±7	169±7	173±8	171±4	--[c]
	corn oil	177±4	--[c]	173±8	177±8	178±8
Organ Weight (g)						
Liver	α-CD	5.73±0.28	5.55±0.31	5.80±0.29	6.94±0.51[d]	–
	Corn oil	5.6±0.56	–	6.10±0.34	8.30±0.60[e]	10.2±1.5[e]
Right Kidney	α-CD	0.67±0.12	0.61±0.02	0.61±0.06	0.69±0.04	–
	Corn oil	0.59±0.04	–	0.62±0.04	0.65±0.07	0.72±0.07[d]
Heart	α-CD	0.74±0.11	0.70±0.06	0.70±0.07	0.72±0.07	–
	Corn oil	0.74±0.05	–	0.78±0.07	0.70±0.06	0.68±0.07
Lung	α-CD	1.07±0.07	1.03±0.04	1.08±0.14	1.11±0.04	–
	Corn oil	1.13±0.09	–	1.10±0.04	1.17±0.20	1.14±0.17
Thymus	α-CD	0.24±0.04	0.25±0.04	0.30±0.04	0.30±0.05	–
	Corn oil	0.22±0.04	–	0.32±0.05[d]	0.28±0.03	0.26±0.06
Organ-to-Body-Weight Ratio (%)						
Liver	α-CD	3.3±0.1	3.3±0.1	3.3±0.1	4.0±0.3[d]	–
	Corn oil	3.2±0.3	–	3.5±0.2	4.6±0.2[d]	5.7±0.6[e]
Right Kidney	α-CD	0.39±0.06	0.36±0.02	0.35±0.03	0.40±0.02	–
	Corn oil	0.34±0.02	–	0.36±0.02	0.36±0.03	0.41±0.04
Heart	α-CD	0.43±0.05	0.42±0.05	0.40±0.05	0.41±0.04	–
	Corn oil	0.42±0.04	–	0.45±0.03	0.40±0.05	0.38±0.05
Lung	α-CD	0.62±0.03	0.61±0.01	0.63±0.07	0.65±0.03	–
	Corn oil	0.64±0.05	–	0.63±0.02	0.66±0.1	0.64±0.09
Thymus	α-CD	0.14±0.02	0.15±0.03	0.17±0.03	0.17±0.03	–
	Corn oil	0.13±0.02	–	0.19±0.03[d]	0.16±0.03	0.15±0.03

a Mean ± standard deviation.
b n= 5 for all dose groups excepting the 1000 mg/kg (corn oil) where 1 rat died during the study.
c No α-CD group at 1000 mg/kg or corn oil group at 10 mg/kg.
d Significantly different from 0 dose group value ($p < 0.01$).
e Significantly different from 0 dose group value ($p < 0.001$).

The α2u-globulin levels in the kidney of male rats after 14 days of dosing with CTFT are shown in Table 9. The results of correlation analysis showed that the average concentration of α2u-globulin in the kidney was strongly associated with dose ($r = 0.987$) (Figure 6). There was no effect of the vehicle on the level of α2u-globulin in the kidney.

At necropsy, livers of male and female and kidneys of males were enlarged in the highest dose group (1000 mg/kg). Treatment-related microscopic changes were seen in these organs. Treatment-related microscopic lesions also were observed in the adrenal gland of both sexes. Both the incidences and severity of the microscopic lesions were dose-related, and there was no apparent effect of dose vehicle (Table 10).

Treatment- and dose-related hypertrophy of hepatocytes was present in the liver of male and female rats administered CTFT with corn oil or α-CD. Hypertrophy was minimal to mild, consisting of enlargement of hepatocytes in the centrilobular region; although cell size was increased, nuclei

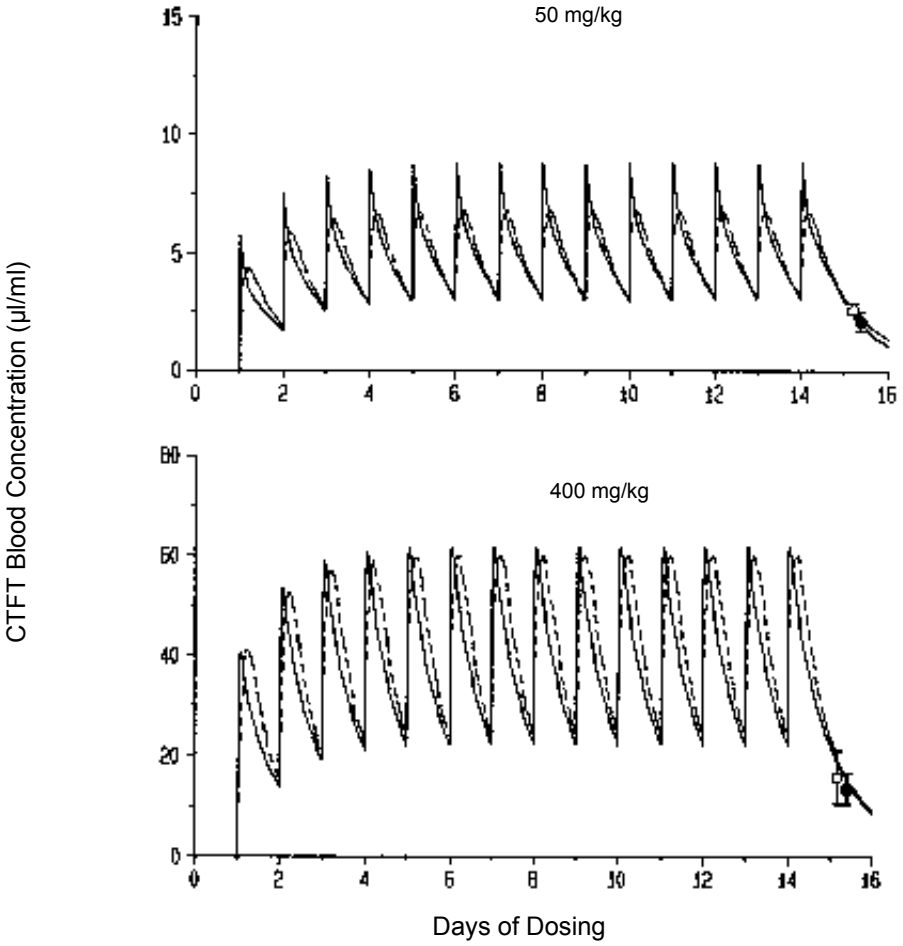

Figure 5

Theoretical CTFT blood concentration profiles in F344/N rats dosed with α-CD(———) or corn oil (-------) vehicle during 14-day studies. The curves were simulated by computer using the previously obtained toxicokinetic parameters. Actual CTFT blood concentrations were determined prior to necropsy in male rats using α-CD vehicle (•) or corn oil vehicle (O).

were not enlarged, and there was no apparent increase in the number of mitotic figures in hepatocytes of treated rats. Rats administered the highest dose of CTFT in corn oil (1000 mg/kg) had the greatest severity of mild hypertrophy, with enlargement of most hepatocytes within the lobule. Ultrastructural changes in hepatocytes from rats with hypertrophy were limited to the cytoplasm and were characterized by increased smooth endoplasmic reticulum when compared to the controls. Lipid droplets in controls generally were smaller and stained less densely than in the dosed rats.

Treatment- and dose-related hypertrophy of hepatocytes was present in the liver of male and female rats administered CTFT with corn oil or α-CD. Hypertrophy was minimal to mild, consisting of enlargement of hepatocytes in the centrilobular region; although cell size was increased, nuclei were not enlarged, and there was no apparent increase in the number of mitotic figures in

TABLE 8 Concentrations of CTFT in Blood, Liver, and Kidney of F344/N Rats after 14 Days of Dosing by Gavage

Dose (mg/kg)	Vehicle	Male			Female		
		Blood (µg/ml)	Liver (µg/g)	Kidney (µg/g)	Blood (µg/ml)	Liver (µg/g)	Kidney (µg/g)
0	α-CD	0	0	0	0	0	0
	corn oil	0	0	0	0	0	0
10	α-CD	0.3±0.2[a]	<3.6[b]	8.5±1	0.3±0.2	<3.6[b]	<3.6[b]
50	α-CD	2.2±0.3	6±1	32±3	2.2±0.3	5.2[c]	3.8[c]
	corn oil	2.5±0.3	9±2	41±7	2.3±0.3	4±1.6	4.8[c]
400	α-CD	20±2	32±7	158±19	20±2	18±2	14±4
	corn oil	16±6	37±7	188±19	20±3	20±9	13±3
1000	corn oil	28±5	50±10.5	257±13	41±11[d]	38±10[d]	29±3[d]

a CTFT concentration expressed as mean ± SD for 5 animals.
b Values below the quantitation limits of 3.6 µg/g.
c One animal value; the values of the other 4 animals were below the quantitation limits of 3.6 µg/g.
d Mean ± SD (4 animals).

TABLE 9 α2u-Globulin in Kidney of Male F344/N Rats After 14 Days of Dosing by Gavage with CTFT[a]

	CTFT Dose (mg/kg)				
Vehicle	**0**	**10**	**50**	**400**	**1000**
α-CD	11±2	9±2	16±2[b]	34±4[b]	--[c]
corn oil	11±1	--[c]	17±1[b]	34±5[b]	55±8[b]

a α2µ-globulin expressed as percent of total protein in kidney homogenate.
b Significantly different ($p < 0.01$; Student t test) from control group.
c Dose not given with this vehicle.

hepatocytes of treated rats. Rats administered the highest dose of CTFT in corn oil (1000 mg/kg) had the greatest severity of mild hypertrophy, with enlargement of most hepatocytes within the lobule. Ultrastructural changes in hepatocytes from rats with hypertrophy were limited to the cytoplasm and were characterized by increased smooth endoplasmic reticulum when compared to the controls. Lipid droplets in controls generally were smaller and stained less densely than in the dosed rats.

Microscopically, toxic nephropathy, dose-related in severity, was present in the kidney of male rats given CTFT (Table 10) (See Plates 1 and 2). A lesion of minimal severity (Grade 1) was present in male rats administered 50 mg/kg in either the corn oil or α-CD vehicle. Morphologically, this change was limited to a slightly increased accumulation of the irregular-shaped protein droplets in the cytoplasm of the tubular cell epithelium. This change was best distinguished from controls in sections stained with Mallory-Heidenhain. A mild (Grade 2) severity of toxic nephropathy was present in several male rats from the 50 and 400 mg/kg dose groups; it consisted of prominent accumulation of intracytoplasmic protein droplets that were clearly evident in H&E stained

TABLE 10 Histopathologic Lesions in F344/N Rats in the 14-day Gavage Studies of CTFT[a]

Dose (mg/kg)	Vehicle	Male			Female	
		Kidney	Liver	Adrenal gland, cortex	Liver	Adrenal gland, cortex
		Toxic nephropathy	Hepatocyte hypertrophy	Cytoplasmic vacuolation	Hepatocyte hypertrophy	Cytoplasmic vacuolation
0	α-CD	0	0	0	0	0
	corn oil	0	0	0	0	0
10	α-CD	0	0	0	0	0
50	α-CD	5 (1.2)[b]	2 (1.0)	0	0	0
	corn oil	5 (1.0)	0	0	0	0
400	α-CD	5 (3.0)	5 (1.0)	5 (1.0)	3 (1.0)	2 (1.0)
	corn oil	5 (2.8)	5 (2.0)	4 (1.0)	5 (1.0)	2 (1.0)
1000	corn oil	5 (3.0)	5 (2.0)	5 (2.0)	5 (2.0)	4 (1.0)

a Five rats per group examined.
b (Average severity score) based on a scale of 1 to 4: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. Scores are averages based on number of animals with lesions.

sections. These droplets were in the tubular epithelium of the cortex and medullary rays of the outer stripe of the outer medulla.

At higher doses, more severe renal lesions (Grade 3) were present. In addition to the accumulation of large, irregular-shaped protein droplets in the epithelium, there was degeneration, necrosis, and regeneration of tubular cells. Eosinophilic granular casts of necrotic cellular debris were present in lumina of distended tubules at the junction of the inner and outer stripe of the outer medulla. There were no treatment-related microscopic changes present in the kidney of male rats administered 10 mg/kg of CTFT in the α-CD vehicle. Electron-microscope examination (Plates 3 and 4) of representative kidney samples from high-dose and control male rats from both α-CD and corn oil groups demonstrated characteristic intracytoplasmic and intraluminal electron-dense protein droplets. The droplets had irregular crystalline shapes that were increased in size and number in treated groups. Tubular cell degeneration was evident by margination of nuclear chromatin, intracytoplasmic vacuolation, and separation of cells from the basal lamina; focal mineralization was present along basal lamina of renal tubules. Kidney weights were slightly increased in female rats dosed with 1000 mg/kg CTFT, but there were no morphologic changes detected by light or electron-microscope examination.

Hematologic analyses showed mild, statistically significant decreases in erythrocyte counts, HGB concentrations, and HCT, and increases in total leukocyte counts (male rats) produced by a mild neutrophilia and monocytosis (Appendix A, Table A1) in male and female rats treated with the highest dose of CTFT (1000 mg/kg) in corn oil. The mild anemias were not accompanied by changes in mean cell volumes (MCV) or mean hemoglobin concentrations (MCH), although reticulocyte counts were significantly increased in female rats. Similar trends in the erythrocyte variables were present in animals treated with CTFT in corn oil and α-CD, but because of the lower doses of CTFT used with the latter vehicle, most changes in hematologic variables in these animals were not significant statistically.

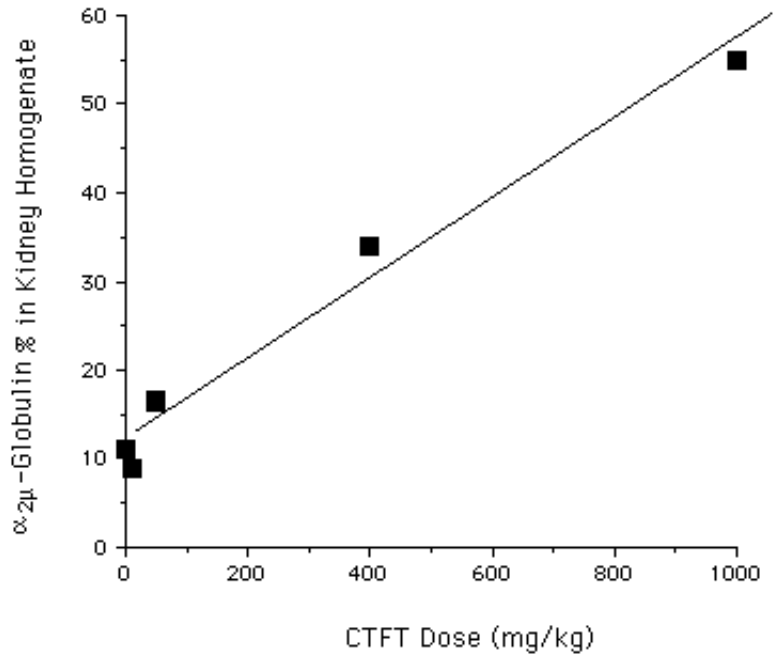

Figure 6
Plot of α2u-globulin in the kidney homogenate vs. CTFT dose.

Significant increases in serum concentrations of cholesterol, triglycerides, total protein, albumin (female rats), and total bile acids and increases in activities of 5'-nucleotidase, ALP (male rats), and SDH (male rats) were seen in animals primarily at the 1000 mg/kg dose with the corn oil vehicle but occasionally at the 400 mg/kg doses with the corn oil or α-CD vehicle (Appendix A, Table A2).

Plates

Plate 1. Proximal tubules of kidney from male F344/N rat treated with 1000 mg/kg CTFT. Many homogeneous, intensely stained, round-to-polygonal-shaped protein droplets (arrows) are present in the cytoplasm of the tubular epithelium. H&E stain, 240X.

Plate 2. Junction of inner and outer stripe of the outer medulla of kidney, from male F344/N rat treated with 1000 mg/kg CTFT. Dilated tubule contains cast (C) of cell debris. H&E, 240X.

Plate 3. Electron photomicrograph showing proximal tubule from kidney of male F344/N rat treated with 1000 mg/kg CTFT. Numerous large, black, irregular-shaped protein droplets (arrows) are present in the cytoplasm of the proximal tubular epithelium. A large cytoplasmic vacuole is present in one tubular cell; other degenerative changes in two cells include margination (M) of nuclear chromatin, blebbing of the cytoplasm into the tubular lumen, and loss of surface microvilli. TEM stain, 2100X.

Plate 4. Proximal tubule from vehicle control male F344/N rat for comparison of morphology of normal protein droplets (arrows) with those from treated rat in Plate 3. TEM, 2100X.

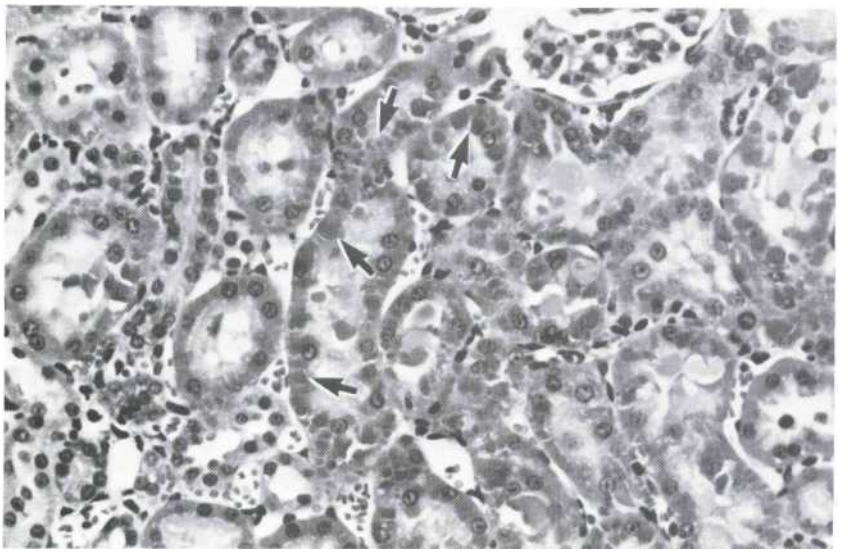

Plate 1

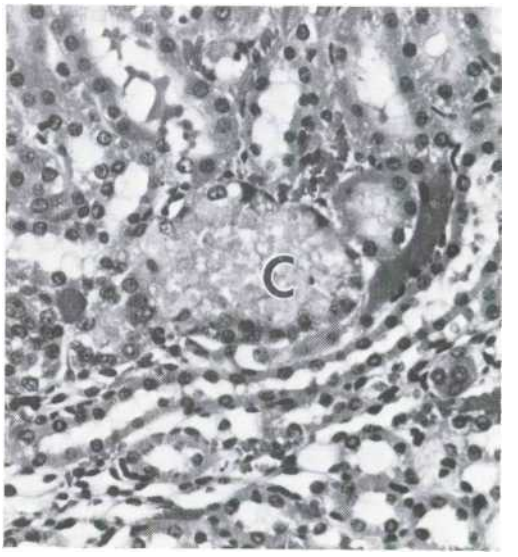

Plate 2

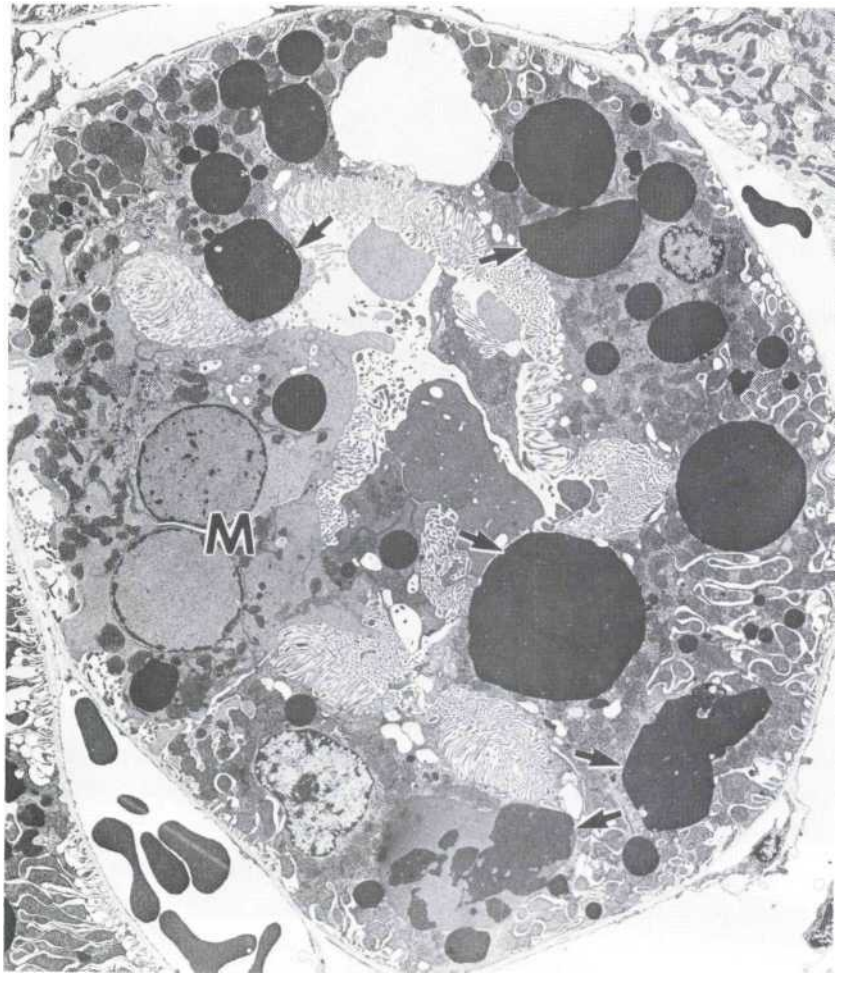

Plate 3

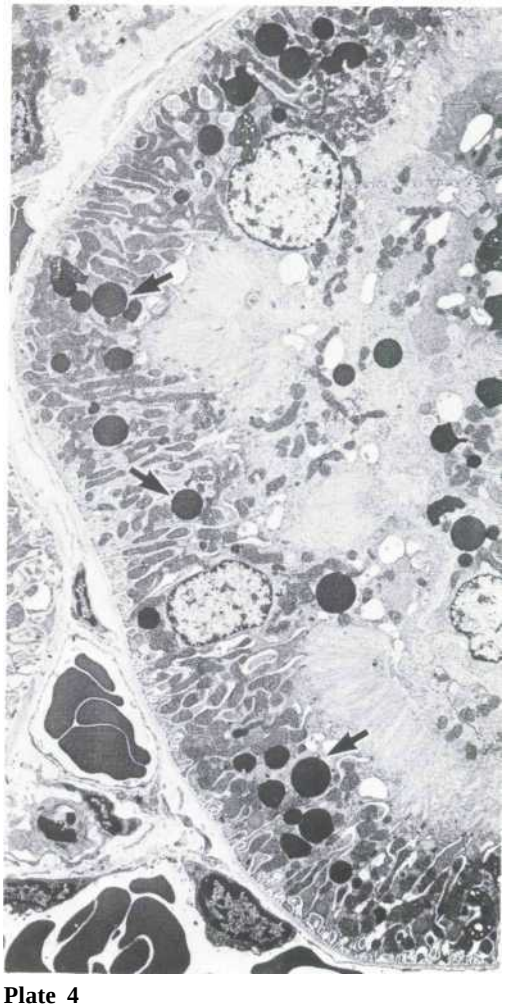

Plate 4

14-Day Toxicity Studies in B6C3F$_1$ Mice

Minimal adverse clinical signs of irritation (burrowing in bedding, rubbing face with forepaws) were seen immediately following dosing with CTFT, regardless of the vehicle used. No treatment-related mortality occurred (Table 11). Body weight gains were not affected by CTFT given in either the corn oil or α-CD vehicle. The only consistent change in organ weight was an increase in liver weight in male (71 percent over control) and female (55 percent over control) mice given 1000 mg/kg CTFT by gavage in corn oil.

TABLE 11 Survival and Weight Gain of B6C3F$_1$ Mice in the 14-Day Gavage Studies of CTFT

Dose Level (mg/kg)	Survival[a]	Mean Body Weight (grams) Initial	Final	Change[b]	Final Weight Relative[c] to Controls (%)
Male					
Corn Oil Vehicle					
0	4/5	27.8	28.9	1.1	
10	5/5	28.4	29.8	1.4	103
50	5/5	29.2	28.1	-1.1	97
400	5/5	27.6	29.6	2.0	102
1000	5/5	29.4	30.1	0.7	104
α-CD Vehicle					
0	5/5	29.6	29.1	-0.5	
10	4/5	28.4	29.0	0.6	100
50	5/5	28.8	28.8	0.0	99
400	5/5	26.2	28.9	2.7	99
Female					
Corn Oil Vehicle					
0	5/5	19.2	21.6	2.4	
10	5/5	20.2	22.1	1.9	102
50	5/5	19.8	21.8	2.0	101
400	5/5	19.5	21.3	1.8	99
1000	5/5	20.0	22.7	2.7	105
α-CD Vehicle					
0	4/5	19.2	21.1	1.9	
10	5/5	19.4	20.6	1.2	98
50	4/5	20.4	21.8	1.4	103
400	5/5	19.6	22.2	2.6	105

a Number surviving at 2 weeks/number of animals per dose group; deaths unrelated to treatment.
b Mean weight change of the animals in each dose group.
c (Dosed group mean/Control group mean) x 100.

When analyzed 24 hours after the last dose of CTFT, blood, liver, and kidney CTFT concentrations in female mice and in most male mice were less than the quantitation limits (3.6 μg/g) even at the highest dose (1000 mg/kg). Only 1 male mouse in the highest dose group was found to have detectable amounts of CTFT in blood (6.5 μg/ml), kidney (3.9 μg/g), and liver (15.4 μg/g). Another male mouse in the same dose group was found to have detectable levels of CTFT in liver (12.4 μg/g) but not in kidney or blood. Compared to the rat data, it is obvious that the elimination of CTFT is much faster in mice.

There were no gross lesions attributed to treatment. Microscopic changes were limited to the liver of dosed male and female mice (Table 12). Hepatocellular hypertrophy was present in mice from

the 400 and 1000 mg/kg dose groups and was slightly more pronounced in males. Hypertrophy was characterized by enlargement of the hepatocytes in the centrilobular area; the cytoplasm of the affected hepatocytes stained less intensely and was less vacuolated than in the liver of controls. In the high dose mice where this lesion was more prominent, the centrilobular zone of hypertrophic hepatocytes extended almost to the periphery of the lobule. At the 400 mg/kg dose level, minimal to mild hepatocyte hypertrophy was present in all male mice, but both the incidence and severity were less in female mice at this dose.

TABLE 12 Liver Hypertrophy in Male and Female B6C3F$_1$ Mice in the 14-Day Studies of CTFT with the Corn Oil or α-CD Vehicle[a]

Dose (mg/kg)	Vehicle	Male	Female
0	α-CD	0	0
	corn oil	0	0
10	α-CD	0	0
	corn oil	0	0
50	α-CD	0	0
	corn oil	0	0
400	α-CD	5 (1.4)[b]	1 (1.0)
	corn oil	5 (1.2)	4 (1.0)
1000	corn oil	5 (2.6)	5 (1.2)

a 5 mice per group
b (Average severity score) based on a scale of 1 to 4: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. Scores are averages based on number of animals with lesions.

Significant increases in serum concentrations of cholesterol and TG and in activities of 5'-nucleotidase were observed in male and female mice treated with CTFT with the corn oil or α-CD vehicle (Appendix A, Table A3). Increases also were seen in concentrations of serum total protein and total bile acids and in activities of ALT and SDH in the 1000 mg/kg corn oil groups.

DISCUSSION

In order to be absorbed, it is assumed that CTFT first must be released from the α-CD complex. This assumption is based on the structure of α-CD, which is very similar to that of β-CD (Szejtli, 1982), and on ^{14}C labelling experiments (Gerloczy *et al.*, 1985) that have shown that β-CD can not be absorbed by rats. Release of CTFT from α-CD can be facilitated in two ways: (1) other molecules that may be present in the gastrointestinal tract compete for the α-CD inclusion site, which is occupied by CTFT; or (2) CTFT is removed rapidly by absorption, which causes a shift in the molecular complex equilibrium, in turn releasing more CTFT. Current studies using α-CD as the dosing vehicle indicate a short time to maximum concentration (T_{max}) and a large absorption rate constant (k_a) for CTFT. This, in turn, suggests that the release and absorption of CTFT from α-CD formulations probably begins immediately in the stomach.

CTFT apparently has a large volume of distribution, as well as a relatively long biological half-life. Both can be predicted from the strong lipophilic nature of the chemical. As expected, these parameters were not influenced by the dosing vehicle. Considering the strong lipophilicity of CTFT, it would be expected that corn oil could act as a depot for the lipid soluble CTFT; it appears from the absorption half-life data that the majority of CTFT was not released until the corn oil was digested. Because corn oil digestion occurs primarily in the intestine, there should be an extended T_{max} for CTFT administered in corn oil, as was confirmed in the current study. The extended T_{max} has been seen in studies with other chemicals (Withey *et al.*, 1983). The existence of this extended time course for release of CTFT from the corn oil vehicle was not considered in the constructed model. This is evident in Figure 2, which shows the T_{max} lags behind that predicted by the model. Incorporating these processes into the model would be unrealistic because it is difficult to construct a simple mathematical description for gastric emptying and digestion (Gladtke and Hattingberg, 1979).

Disposition studies of CTFT labelled with ^{14}C in the trifluoromethyl group showed that when male Sprague-Dawley rats were given a single oral 1 mg/kg dose of CTFT in corn oil, 62 - 80% of CTFT was expired, unchanged, from the lung within 24 hours. Only a small amount of conjugated CTFT was found in the urine. Peak levels of CTFT eliminated via the lung occurred about 6 - 7 hours after dosing (Quistad and Mulholland, 1983). This is consistent with our finding of a 7.6 hour T_{max} for CTFT in corn oil.

Because the lung is the major organ responsible for the clearance of CTFT (Quistad and Mulholland, 1983), a pulmonary first-pass effect would be expected (Collins and Dedrick, 1982). If this first-pass effect existed, the AUC/dose would be lower after an i.v. dose than after an oral dose, because the elimination of CTFT from the lung is a first-order process. The amount of CTFT cleared by the lung is directly proportional to the CTFT concentration. In i.v. administration the concentration in the venous blood is very high at first, resulting in the larger loss of CTFT in the lung. In the case of the oral route, the venous blood concentrations rise gradually, resulting in a smaller loss of CTFT than would be expected after i.v. dosing. In fact, in the current study the average AUC/dose value obtained after i.v. administration was significantly lower ($p < 0.01$) than after gavage, with either the α-CD or corn oil vehicle, which indicates a pulmonary first-pass effect is occurring.

When the AUC is underestimated because of the first-pass effect, the V_d and CL_T would be correspondingly higher because they are inversely related. This explains why V_d and CL_T from the i.v. data (13.1 L/kg and 7.8 ml/min/kg, respectively) are larger than from the gavage data (11.9 L/kg and 6.7 ml/min/kg).

Two dosing formulations are considered to be bioequivalent if their rates and extents of systemic availability are the same (Cabana, 1977). The current study shows that the α-CD and corn oil formulations are not bioequivalent, since k_a and T_{max} are vehicle dependent; but the extent of systemic availability is the same since AUC is vehicle independent. A similar toxicologic response for CTFT should be expected, given that the dose-normalized C_{max} is similar for all doses and both vehicles, that systemic availability is present, and that CTFT is a weakly toxic chemical, based on its LD_{50} value. A similar toxicologic response for CTFT should be expected. These predictions are supported by the results of these 14-day studies using male and female Fisher 344/N rats and $B6C3F_1$ mice.

Previous studies showed that CTFT can cause a toxic (hyaline droplet) nephropathy and an increase of relative kidney and liver weight at the 1000 mg/kg dose level in rats (Macri *et al.*, 1987). Results of the current study are consistent with those reported. These results also showed that the morphologic appearance of hyaline droplets in the kidney correlated with the increase of α2u-globulin, reportedly unique to some strains of male rats. Both male and female rats showed an accumulation of CTFT in the liver and kidney. In liver, the extent of accumulation was in the same range for both sexes, although accumulation in male rats was slightly higher than in females. The accumulation of CTFT in the kidney of male rats, however, was much greater than in female rats. This finding may account for the more severe kidney damage in male rats and the difference in kidney weight increases between the sexes.

The major accumulation of CTFT in the male rat kidney probably was caused by the reversible binding of CTFT to α2u-globulin; there have been several reports of specific, reversible binding of the α2u-globulin with a variety of hydrocarbons or their metabolites, including 2,2,4-trimethylpentane, limonene, and decalin (Borghoff *et al.*, 1988; Swenberg *et al.*, 1989; Ridder *et al.*, 1990). Since α2u-globulin is synthesized in the liver, the reversible binding of α2u-globulin with CTFT should also occur in the liver. This may explain why liver concentrations of CTFT in male rats are higher than in female rats. The accumulation of α2u-globulin in male rat kidney has been associated with hydrocarbon-induced proximal tubular cell degeneration. Morphologic changes (observed by light and electron microscopy), typical of those described for "hydrocarbon" or "hyaline droplet" nephropathy, were present in male rats; all groups receiving CTFT at 50 mg/kg and higher were affected, but no morphological changes were seen in a low dose group of 10 mg/kg. Slight increases in kidney weights were also noted in the higher dose groups in males (400 and 100 mg/kg). The increased weights corresponded to the severity grade of toxic nephropathy. Increased liver weights also were noted, likely due to the hypertrophy of hepatocytes and proliferation of smooth endoplasmic reticulum. Cytoplasmic vacuolation of the adrenal gland was slightly more prominent in males. The cause or biological significance of the adrenal change is not clear. In toxicity studies, microscopic changes (vacuolation, hypertrophy, lipidosis, etc.) are often observed in the zona reticularis of the adrenal cortex (Rebelin, 1984). The adrenal gland may be subject to direct chemical injury, and the lipophilic nature of the adrenal may result in the accumulation of

hydrophobic compounds. It was not possible to determine in the current study if the observed change was the result of stress, or of the increase of ACTH stimulation due to the impaired adrenal steroid production, or of other mechanisms.

CTFT treatment produced mild anemias in both male and female rats. In females, the increase in absolute reticulocyte counts provided evidence that the anemia was regenerative. This finding is consistent with decreased life spans of circulating erythrocytes produced by CTFT and with a compensatory release of young red cells (reticulocytes) from the bone marrow. Additional evidence for this process, such as increases in MCV and MCH, was not present, possibly because of the mild nature of the effect. In male rats, however, there was no evidence of an enhanced regenerative effect associated with the anemia. If the cause of the anemia in male rats was similar to that suggested for female rats, a regenerative response should have developed. Several possibilities can be proposed. For one, if the causes of the anemias are the same, the renal disease that occurred in male but not female rats (Table 10) may have compromised the ability of the male rats to produce erythropoietin. This could have resulted in a mild, poorly regenerative anemia.

Predominant findings from the clinical pathology studies included increases in concentrations of cholesterol and in activities of 5'-nucleotidase, both in rats and mice. Although not as consistent as these effects, increases also occurred in concentrations of triglycerides, total bile acids, and total protein. Together, these changes support the histopathologic findings of hypertrophy and provide clear evidence of an hepatic effect. Increases in cholesterol, TG, 5'-nucleotidase, and total bile acids are associated with cholestasis, an impaired production or flow of bile (Zakim and Boyer, 1982). These biochemical changes were not accompanied by histopathologic evidence of hepatocellular toxicity; this indicates a cellular mechanism of cholestasis, rather than a physical one. Cellular mechanisms which might account for these effects include CTFT-induced alterations in plasma membranes (function and composition), disruptions in microfilaments and microtubules, loss of tight junction integrity, and/or changes in the concentration and composition of bile acids.

The faster elimination of CTFT in mice than in rats can be accounted for by differing metabolic and respiration rates, and/or by differences in lung surface area as a function of body weight. The lung was identified as the major organ of elimination for CTFT in rats. If this also is the case in mice, it suggests the allometric equation, $t_{1/2} = A(W^{0.75})$ (Yacobi *et al.*, 1989), where A is constant and W is the body weight of the animal. (The choice of the exponent of 0.75 in the equation is based upon the fact that the lung is the main organ of elimination.) The equation can be used to estimate the half-life of CTFT in mice (approximately 4 hours) based on the half-life of CTFT in rats (approximately 20 hours). The sampling time for blood and tissues was about 24 hours after the last dose in the current study. This was equal to 6 times the calculated CTFT half-life in mice. At this time, practically all the CTFT would be eliminated, thus accounting for the lack of measurable levels of CTFT in blood or tissue of mice.

The dosing vehicle used had no significant effect on organ weight changes, on the accumulation of CTFT in the liver and kidneys of either sex of rats, or on the α2u-globulin accumulation in male rat kidneys. The data from these studies strongly support the conclusion that the use of the α-CD vehicle had no effect on the toxicity of CTFT; therefore α-CD is an acceptable vehicle for toxicity studies with CTFT and may find general use in other toxicology studies.

Based on the data obtained in these studies, CTFT might be expected to induce renal tubular cell tumors in male F344/N rats in long term studies, as has been shown with other chemicals which induce an accumulation of α2u-globulin in the kidney (Swenberg *et al.*, 1989). CTFT does not appear to be a mutagen, and there is little reason to suspect that it would be genotoxic. However, this does not exclude the possibility that CTFT may be a carcinogen in mice or female rats, or at sites in male rats in addition to the kidney.

In conclusion, CTFT was found to be mildly toxic to F344/N rats and B6C3F_1 mice in 14-day oral administration studies. The rat was found to be somewhat more sensitive to the toxic effects of CTFT than the mouse, likely because of the faster rate of elimination from the mouse than the rat. A daily dose of 10 mg/kg appeared to be a no-observed-adverse-effect level for male rats; higher doses resulted in hepatocyte hypertrophy and a toxic nephropathy associated with α2u-globulin accumulation. Hepatocyte hypertrophy and adrenal gland cytoplasmic vacuolation occurred in female rats at 400 mg/kg and higher doses. Hypertrophy of hepatocytes also occurred in mice given 400 mg/kg and higher doses, but other adverse effects were not noted in mice.

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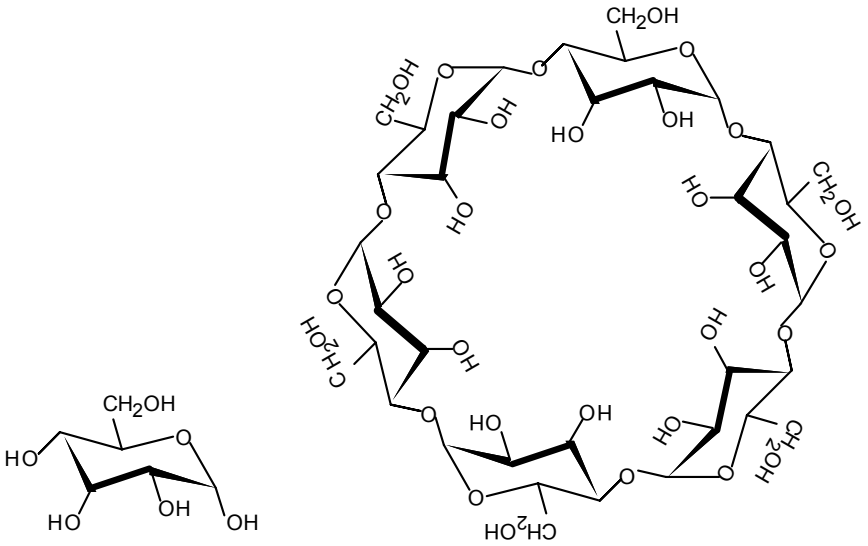
APPENDIX A

CYCLODEXTRIN AND MOLECULAR COMPLEXATION

Introduction

Cyclodextrins, sometimes called cycloamyloses, are a series of oligosaccharides produced by the action of amylase of *Bacillus macerans* on starch (Bender and Komiyama, 1978). Industrial production of cyclodextrins has been achieved in Japan, Hungary and USA. Structurally, all cyclodextrins have doughnut-shapes and are composed of α-(1,4)-linkages of a number of D(+)-glucopyranose units (Figure1). Greek letters are used to denote the number of glucose units: α- for 6, β- for 7, γ- for 8 and so on.



D(+)-glucose **α-Cyclodextrin**

Figure 1

Chemical structure of D(+)-Glucose and α-cyclodextrin.

Cyclodextrin cavities are slightly "V" shaped with the secondary hydroxyl side more open than the primary hydroxyl side. The interior of the cavity consists only of a ring of C-H groups, a ring of glucosidic oxygens, and another ring of C-H groups; therefore, the interior of the cavity is similar to the lipophilicity of diethyl ether (Figure 2). The dimension of the cylodextrin cavity depends on the number of glucose units in it. The more glucose units there are, the bigger the cavity is. The cavity dimensions for β-cyclodextrin (β-CD) are 7.0 Å (internal diameter) and 7.0 Å (cavity depth); for α-cyclodextrin (α-CD), the dimensions are 4.5 Å (internal diameter) and 6.7 Å (cavity depth), respectively. Hence, the cavity of the β-CD is larger than that of the α-CD. The molecular weights of α-CD and β-CD are 972 and 1135 Daltons, respectively.

In aqueous solution, the slightly apolar CD cavity is occupied by water molecules that are energetically unstable because of the polar-apolar interaction. These water molecules can be readily replaced by appropriate "guest molecules," which are less polar than water molecules, to form the more energetically stable complexes (Figure 3).

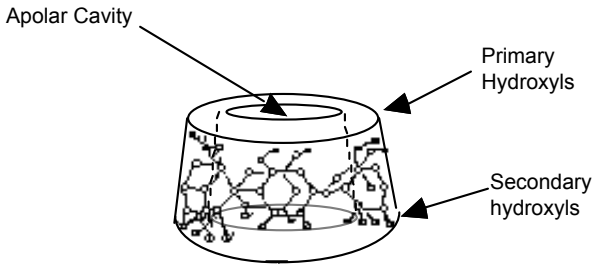


Figure 2

Schematic structure of α-cyclodextrin.

Many water-insoluble organic chemicals similar in size to a benzene or naphthalene ring, or even larger ones which have a side chain of comparable size, can be trapped in cyclodextrin cavities by this mechanism. This process is called "molecular inclusion," or "molecular complexation." Generally, molecular complexation enhances the water solubility of the resulting organic chemical. If the concentration of the inclusion complex exceeds the saturation point, however, precipitation of the stable complex will take place (Szejtli, 1982).

The cyclodextrins vary in water solubility. γ-CD is the most water soluble (23.3 g/100ml); α-CD is next most soluble (14.5 g/100ml); and β-CD is the least soluble (1.85 g/100ml). Because of its high cost, γ-CD is used rarely in research; α-CD and β-CD are the most widely used of the cyclodextrins. It is widely accepted that the the molecular complex of β-CD is much more stable than α-CD, owing to β-CD's larger cavity. The low solubility of β-CD, which limits its application, can be enhanced greatly by chemical modification (Szeman *et al.,* 1987a, 1987b; Pitha and Pitha, 1985). Some of these products are commercially available and are considered non-toxic (American Maize Products Company, Hammond, Indiana), although further toxicity studies are needed before they can be considered for use in pharmaceuticals in the United States.

Because α- and β-CD are non-toxic when taken orally, they have been widely used as flavor-preserving agents in the food and cosmetic industries, such as in masking bitterness in grapefruit juice and the unpleasant odor of fish bone in animal food, and in preserving the flavor of tea, etc. (Szejtli *et al.*, 1980; Fukinbara, 1980). Research on cyclodextrins has progressed rapidly in recent years, and studies of pharmaceutical applications is a very active area at present. The Japanese

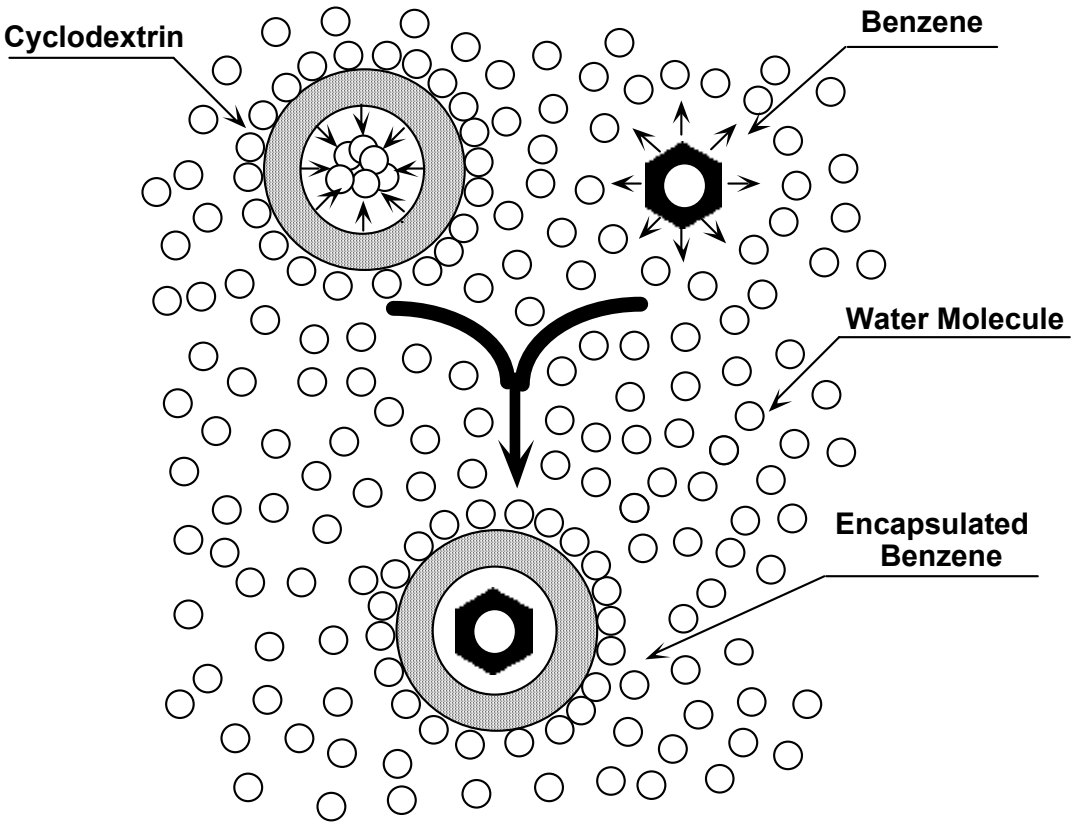


Figure 3

Schematic illustration of molecular complexation of benzene. The water molecules inside the cyclodextrin are replaced by benzene; the driving force is a polar-apolar interaction.

government has authorized the therapeutic use of drugs containing cyclodextrins, including prostaglandin E_2-β-CD, alprostadil-α-CD, limaprost-α-CD (Ono Pharmaceutical, Japan), and benexate hydrochloride-β-CD (Shionogi, Japan). Italian government drug authorities have registered iroxicam-β-CD, developed by Chiesi (Koizumi *et al.,* 1988).

Cyclodextrins are considered to offer the following advantages in the manufacture of pharmaceuticals:

- Liquid compounds can be transformed into crystalline or non-crystalline forms, for the manufacture of tablets.
- Volatile compounds can be stabilized against evaporation losses.
- Compounds oxidizable in air can be protected by CD-complexation.
- Unpleasant tastes or smells of some drugs can be masked with CDs.
- Incompatible drugs can be mixed together if one of them is protected specifically by CD.
- The water solubility of a drug, as well as the dissolution rate of poorly soluble drugs, can be increased.
- Following oral administration of a drug with low water-solubility, higher blood levels can be achieved if the drug is complexed with CD.

There also are a few limitations in using cyclodextrins in pharmaceuticals:

- Not all drugs can be molecularly complexed with CD.
- If a drug's molecular weight is small, the capacity of the drug for molecular complexation is limited, because of the large molecular weight of cyclodextrin (about 1000), and because the complexation ratio (drug:CD) usually must be 1:1, 1:2, or 3:2.

Increased incidences of pancreatic lesions observed in male F344/N rats that were administered corn oil by gavage (Boorman and Eustis, 1985; Eustis and Boorman, 1985; Haseman *et al.*, 1985) indicated that alternative dosage vehicles should be investigated. Microencapsulation technology was introduced in previous toxicology studies (Melnick *et al.*, 1987a, 1987b), and volatile chemicals such as trichloroethane, 2-ethylhexanol, cinnamaldehyde, and citral were successfully encapsulated using a starch-and-geletin mixture as the shell material. The development and use of microcapsulated chemicals that can be mixed in feed as an alternative to corn oil gavage dosing (Melnick *et al.,* 1987a, 1987b) has been successful; the current investigation of molecular complexation technology, in this case with CTFT, is a logical extension of these efforts. Results of the formulation stability, syringeability, and vehicle load capacity of cyclodextrin with CTFT are reported here.

Methods

Colorimetric Analysis of Molecularly Complexed CTFT

Concentrations of CTFT in the supernatants and precipitates of molecularly complexed CTFT aqueous suspensions were measured colorimetrically. The supernatants were filtered with a Millex-SR Millipore filter (Millipore Corporation, Bedford, MA); the absorbance at 265 nm was determined using a Hewlett-Packard 8450A diode array spectrophotometer (Hewlett-Packard Corporation, Palo Alto, CA) with 13.6% aqueous α-CD as the reference. For determination of CTFT in the precipitates, an aliquot of the precipitate was weighed, dissolved in 10 ml of a mixed solvent (1:1 water/isopropanol), and the absorbance determined using the same mixed solvent as the reference sample. Standard solutions were prepared by adding CTFT (2, 4, 8, 10, 16, and 20 µl) directly into 50 ml of the solvent to produce 6 standard solutions of CTFT (0.027, 0.054, 0.108, 0.159, 0.216, and 0.270 mg/ml). Concentrations of CTFT in the supernatants and in precipitates were calculated from a standard curve.

Results

Properties of Molecularly Complexed CTFT

No significant change in the UV spectrum of CTFT occurred after it was molecularly complexed (Figure 4), indicating that a colorimetric analysis method for complexed CTFT could be developed. To determine the enhancement of solubility of CTFT in water after molecular complexation with α-CD, approximately 1 ml of CTFT was added to 50 ml aliquots of different concentrations of α-CD

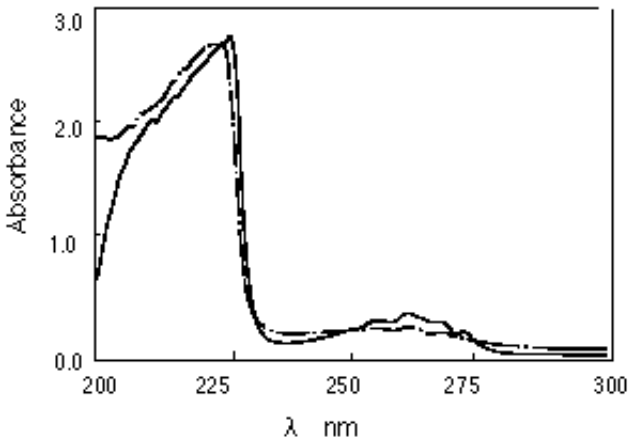


Figure 4

U.V. Spectra 1:1 water/isopropanol solution of CTFT and α-CD molecular complexed solution of CTFT. (———) neat CTFT; (— • —) α-CD molecular complexed CTFT

solutions (up to 13.6% w/v). The mixtures were stirred for 3 days at room temperature and allowed to stand overnight. A portion of the supernatant was analyzed using the spectrophotometric procedure. An increase in water solubility of CTFT was found as the concentration of α-CD increased (Figure 5). However, the increased solubility (0.35 mg CTFT/ml) still was not high enough to permit the use of drinking water as a dosage vehicle for administering the test chemical, because of the anticipated low toxicity of CTFT.

In order to determine the utility of the α-CD precipitated complex of CTFT in the preparation of feed formulations, the stability of the complex was determined. The above suspensions were filtered with a Nalgene® filter, and the precipitate was allowed to dry in a hood in an open beaker for a week. A portion of the dried precipitate was analyzed by dissolving it in a mixture of equal volumes of water and isopropanol. Initially, the weight percent of CTFT in the precipitate was 9%. After three weeks, the amount of CTFT decreased to 7%, indicating a 22% loss of CTFT. Although the CTFT in the dried molecular complexed form is much less volatile than the neat chemical, it would appear that the dry CTFT complex still would not be suitable for incorporating into the diet of rodents.

In order to assess the enhancement of CTFT solubility by β-CD, suspensions were prepared by adding approximately 0.5 ml of CTFT to 50 ml aliquots of different concentrations of β-CD solutions (up to 1.8%, w/v). Results of analysis of the supernatant showed only a very small increase in CTFT solubility which could be explained by the lower water solubility of β-CD (Figure 5).

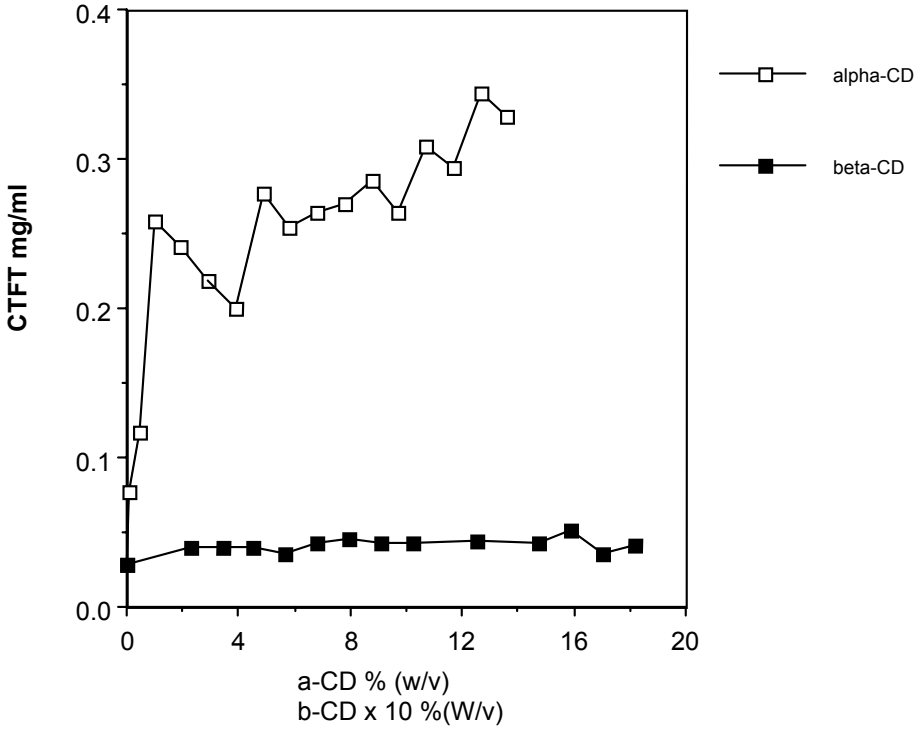


Figure 5

Enhancement of water solubility of CTFT after molecular complexation by aqueousα-CD or β-CD solutions at different concentrations.

A stability study of the precipitated inclusion complex of CTFT with β-CD was conducted as described above for α-CD. Initially, the volume of CTFT in the precipitate, by weight, was 12%; this was very close to the theoretically calculated 13.7% based on an assumed 1:1 ratio for the molecular complexation. After 3 weeks, the volume decreased to 9.3%. Therefore, the β-CD precipitate was not considered suitable for dosing rodents in a feed formulation.

It was not possible to prepare aqueous solutions of molecularly complexed CTFT suitable for dosed water use, nor could precipitates of molecular complexed CTFT be prepared that were suitable for dosed feed. Thus, an effort was made to prepare CTFT/CD suspensions for the gavage route.

Gas Chromatographic Analysis of α-CD/CTFT Suspensions

Normally, CTFT that has been molecularly complexed in α-CD has a relatively low solubility in water, which means that large volumes of isopropanol/water solvent are needed to dissolve the complex for analysis by spectrophotometry. This problem was resolved by development of a method for extracting CTFT from the suspension with an organic solvent. Initial efforts, using hexane or diethyl ether as extracting solvents, resulted in the formation of new white suspensions which actually were the inclusion complexes of the extracting solvents themselves. This molecular

complexation process could be interrupted, however, by first adding a strong basic solution (0.5 N NaOH) to the CTFT inclusion-complex suspension, then adding hexane as the extracting solvent. The clear hexane extract then was mixed with tridecane internal standard and analyzed by gas chromatography (GC).

Six different concentrations of CTFT suspension were prepared individually by adding 0.05, 0.10, 0.20, 0.40, 0.60, and 0.80 ml CTFT into 50 ml aliquots of a 13.6% α-CD aqueous solution and stirring for 3 days. The low and high concentrations were prepared in triplicate to allow estimation of the standard deviation of the whole preparation and analysis procedure. Equivalent solutions of neat CTFT/hexane were prepared and analyzed to determine the absolute recovery from the CTFT suspensions. Recovery for 9 samples averaged 101% with the standard deviations at the low and high concentrations of 3% and 4%, respectively.

Stability of the Suspension

A 500 ml α-CD suspension of molecularly complexed CTFT, containing 21.4 mg/ml CTFT, was prepared according to the previously described method. Five 50 ml portions of the suspension were pipetted into septum vials while the suspension was magnetically stirred. One vial was analyzed immediately. The other 4 vials were sealed; 2 were stored at room temperature, the other 2 at 5°C. The vials were removed and the contents resuspended and analyzed after storage for 14 and 21 days. Additional vials were exposed to air while their suspensions were continuously stirred for 3 hours to simulate actual animal room dosing conditions. The results (Table 1) showed there was no loss of CTFT after 21 days' storage or during handling under simulated dosing room conditions.

TABLE 1 Recovery of CTFT After Storage of Molecularly Complexed Suspensions

Storage Time and Temperature	CTFT Theoretical (mg/ml)	CTFT Found (mg/ml±SD)[a]	Recovery (%)
Zero-time (room temp.)	21.4	20.9±0.4	98
3 hrs open (room temp.)	21.4	22.0±0.4	102
14 days (room temp.)	21.4	22.1±0.0	103
14 days (5°C)	21.4	21.4±0.7	100
21 days (room temp.)	21.4	21.8±0.3	102
21 days (5°C)	21.4	21.9±0.3	102

[a] SD = Standard Deviation

Homogeneity of the Suspension

A set of 1000 ml CTFT/α-CD suspensions were prepared at concentrations which would provide doses for rats and mice of 10, 50, and 400 mg/kg. Samples were taken for analysis from the bottom, middle, and top of the stirred suspension. Table 2 presents the results of the GC analysis of these suspensions. Based on the excellent precision of the analyses at each concentration, it can be concluded that the prepared suspensions were homogeneous.

Syringeability Study

A syringeability study showed the above suspensions could be delivered easily through a 22-gauge gavage needle. GC analysis of samples taken with a plastic syringe equipped with 16- and 22-gauge gavage needles gave reproducible results with relative standard deviations well below 10% (Table 3). Because surface tension of the suspensions was significantly lower than that of water, disposable, gas-tight plastic syringes were preferred for dose administration instead of glass syringes.

TABLE 2 Gas Chromatographic Analysis of Dose Formulations of Molecularly Complexed CTFT Suspensions

CTFT Conc. (mg/ml)	Found (mg/ml±SD)	Recovery (%)
1	0.97±0.01	97
2	1.9±0.1	96
5	5.02±0.04	100
10	9.9±0.1	99
40	40.7±0.06	101
80	79.5±0.5	9

TABLE 3 Syringeability Study of Molecularly Complexed CTFT Suspensions (n=3)

CTFT Concentration (mg/ml)	Syringe Size (ml)	Needle (guage)	Sampling Volume (ml)	RSD (%)
50	1	16	1.0	3[a]
50	3	16	1.0	2
50	1	22	0.2	6
80	1	22	0.2	2

a n=10

Discussion

Based on the available literature, β-CD is capable of forming more stable molecular complexes (1:1 molar ratio) than α-CD, because of its larger cavity (Aboutaleb *et al.,* 1986). Results presented here are consistent with the literature in that the initial percentage of CTFT in the β-CD-complexed precipitate, on a weight basis (12%), was very close to the theoretical value of 13.7%, based on a 1:1 molar ratio complex. For α-CD the percentage of CTFT (9%) was much less than that which would be present in the theoretical 1:1 molar ratio complex (15%). However, due to the low solubility of β-CD in water (1.85 g/100 ml), it would have been difficult to prepare CTFT inclusion-complex suspensions with β-CD at concentrations suitable for toxicity studies. α-CD has a much higher solubility in water and, despite its lower affinity for CTFT, it was still possible to obtain higher concentrations of stable CTFT inclusion-complex suspensions.

Molecular complexation of chemicals in cyclodextrins offers several advantages over microencapsulating such chemicals in a starch-and-gelatin-mixture shell material (Melnick *et al.,* 1987a, 1987b). Because molecular complexation does not require sophisticated equipment and expertise, so the cost of dose formulation is reduced. The resulting molecularly complexed chemicals can be formulated as water solutions, water suspensions, or solid precipitates, depending on the chemicals. These formulations can be incorporated into drinking water or feed. There are disadvantages, however: Some volatile chemicals cannot be molecularly complexed with CD; and the solubility of resulting molecularly complexed chemicals often remains lower than the required concentration for drinking water studies. Also, molecular complexation allows a loading capacity for chemicals that ranges only between 10 - 25% of their weight. Microencapsulation with starch and gelatin offers an advantage in that its loading capacity can be 40 - 80% on a weight basis, and it can be used, in theory, to encapsulate virtually all liquid or solid chemicals. Given the the authorization by Italian and Japanese government authorities for thereapeutic use of drugs molecularly complexed in α-CD or β-CD, the use of these cyclodextrins as dosage vehicles in toxicology studies also should be acceptable.

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APPENDIX B

Hematology and Clinical Chemistry

TABLE B1 Hematology Data for F344/N Rats in the 14-Day Gavage Study of *p*-Chloro-α,α,α-trifluorotoluene[1]

Analysis	0 mg/kg	10 mg/kg	50 mg/kg	400 mg/kg	1000 mg/kg
MALE					
n	5	5	5	5	5
Hematocrit (%)					
Corn oil	49.9 ± 0.3	—[2]	49.3 ± 1.0	47.3 ± 0.6*	46.6 ± 0.6**
α-Cyclodextran	47.7 ± 0.5	48.1 ± 0.5	49.3 ± 0.7	46.2 ± 0.4	—
Hemoglobin (g/dL)					
Corn oil	15.6 ± 0.2	—	15.5 ± 0.3	14.9 ± 0.1*	14.7 ± 0.2**
α-Cyclodextran	15.3 ± 0.1	15.5 ± 0.1	15.7 ± 0.2	14.6 ± 0.0	—
Erythrocytes (10^6/μL)					
Corn oil	9.41 ± 0.08	—	9.26 ± 0.19	8.87 ± 0.14*	8.82 ± 0.10**
α-Cyclodextran	9.02 ± 0.08	9.02 ± 0.08	9.31 ± 0.11	8.65 ± 0.08	—
Reticulocytes (10^6/μL)					
Corn oil	0.17 ± 0.02	—	0.17 ± 0.02	0.17 ± 0.02	0.15 ± 0.00
α-Cyclodextran	0.21 ± 0.01	0.15 ± 0.02	0.15 ± 0.02	0.18 ± 0.02	—
Mean cell volume (fL)					
Corn oil	53.2 ± 0.4	—	53.4 ± 0.2	53.4 ± 0.5	52.6 ± 0.2
α-Cyclodextran	53.0 ± 0.5	53.4 ± 0.2	52.8 ± 0.2	53.4 ± 0.2	—
Mean cell hemoglobin (pg)					
Corn oil	16.6 ± 0.2	—	16.7 ± 0.1	16.9 ± 0.1	16.6 ± 0.1
α-Cyclodextran	16.9 ± 0.1	17.1 ± 0.1	16.9 ± 0.0	16.9 ± 0.2	—
Mean cell hemoglobin concentration (g/dL)					
Corn oil	31.3 ± 0.2	—	31.5 ± 0.1	31.6 ± 0.2	31.5 ± 0.2
α Cyclodextran	32.0 ± 0.1	32.2 ± 0.1	32.0 ± 0.1	31.6 ± 0.3	—
Platelets (10^3/μL)					
Corn oil	696.2 ± 20.2	—	715.6 ± 15.9	716.8 ± 51.7	713.8 ± 36.2
α Cyclodextran	747.8 ± 26.2	715.4 ± 15.0	741.6 ± 29.0	849.0 ± 26.0	—
Leukocytes (10^3/μL)					
Corn oil	7.86 ± 0.34	—	7.44 ± 0.55	8.58 ± 0.66	10.34 ± 0.61*
α Cyclodextran	8.32 ± 0.76	8.02 ± 0.47	8.62 ± 0.42	8.78 ± 0.32	—
Segmented neutrophils (10^3/μL)					
Corn oil	1.51 ± 0.08	—	1.53 ± 0.05	2.04 ± 0.15*	2.27 ± 0.19**
α Cyclodextran	1.78 ± 0.17	1.68 ± 0.14	1.78 ± 0.15	2.07 ± 0.11	—
Lymphocytes (10^3/μL)					
Corn oil	5.53 ± 0.30	—	5.22 ± 0.49	5.48 ± 0.50	6.81 ± 0.30
α Cyclodextran	5.48 ± 0.44	5.47 ± 0.54	5.81 ± 0.38	5.67 ± 0.32	—
Activated lymphocytes (10^3/μL)					
Corn oil	0.48 ± 0.03	—	0.39 ± 0.03	0.60 ± 0.10	0.74 ± 0.08
α Cyclodextran	0.63 ± 0.12	0.48 ± 0.04	0.61 ± 0.06	0.60 ± 0.04	—
Monocytes (10^3/μL)					
Corn oil	0.21 ± 0.02	—	0.21 ± 0.02	0.28 ± 0.04	0.43 ± 0.05**
α-Cyclodextran	0.27 ± 0.05	0.27 ± 0.01	0.31 ± 0.03	0.32 ± 0.02	—
Basophils (10^3/μL)					
Corn oil	0.04 ± 0.00[3]	—	0.04 ± 0.01	0.07 ± 0.01	0.06 ± 0.02
α Cyclodextran	0.04 ± 0.00	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	—
Eosinophils (10^3/μL)					
Corn oil	0.06 ± 0.00	—	0.05 ± 0.01	0.05 ± 0.01[3]	0.05 ± 0.01
α Cyclodextran	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	—

TABLE B1 Hematology Data for F344/N Rats in the 14-Day Gavage Study of *p*-Chloro-α,α,α-trifluorotoluene (continued)

Analysis	0 mg/kg	10 mg/kg	50 mg/kg	400 mg/kg	1000 mg/kg
FEMALE					
n	5	5	5	5	4
Hematocrit (%)					
Corn oil	47.7 ± 0.7	—	47.0 ± 0.8	46.2 ± 0.4	44.3 ± 0.5**
α-Cyclodextran	47.1 ± 0.6	47.4 ± 0.3	47.7 ± 0.6	46.6 ± 0.5	—
Manual hematocrit (%)					
Corn oil	47.2 ± 0.7	—	46.6 ± 0.8	46.8 ± 0.4	44.5 ± 0.7*
α-Cyclodextran	47.2 ± 0.9	47.2 ± 0.5	47.6 ± 0.4	46.8 ± 0.4	—
Hemoglobin (g/dL)					
Corn oil	15.3 ± 0.2	—	15.2 ± 0.3	14.9 ± 0.1	14.2 ± 0.2**
α-Cyclodextran	15.5 ± 0.2	15.3 ± 0.1	15.5 ± 0.2	15.0 ± 0.1*	—
Erythrocytes (10^6/μL)					
Corn oil	8.46 ± 0.13	—	8.37 ± 0.13	8.18 ± 0.05	7.91 ± 0.11**
α-Cyclodextran	8.56 ± 0.10	8.47 ± 0.06	8.54 ± 0.11	8.26 ± 0.08	—
Reticulocytes (10^6/μL)					
Corn oil	0.13 ± 0.01	—	0.17 ± 0.03	0.19 ± 0.02	0.28 ± 0.03**
α-Cyclodextran	0.13 ± 0.02	0.15 ± 0.02	0.14 ± 0.03	0.18 ± 0.02*	—
Mean cell volume (fL)					
Corn oil	56.6 ± 0.2	—	56.2 ± 0.2	56.8 ± 0.2	56.5 ± 0.3
α-Cyclodextran	55.2 ± 0.5	56.0 ± 0.0	56.0 ± 0.0	56.6 ± 0.2**	—
Mean cell hemoglobin (pg)					
Corn oil	18.1 ± 0.1	—	18.1 ± 0.1	18.2 ± 0.1	17.9 ± 0.0
α Cyclodextran	18.2 ± 0.1	18.1 ± 0.1	18.2 ± 0.1	18.1 ± 0.1	—
Mean cell hemoglobin concentration (g/dL)					
Corn oil	32.1 ± 0.3	—	32.3 ± 0.1	32.3 ± 0.1	31.9 ± 0.1
α Cyclodextran	33.0 ± 0.3	32.3 ± 0.2	32.5 ± 0.0	32.1 ± 0.2*	—
Platelets (10^3/μL)					
Corn oil	688.0 ± 22.1	—	741.4 ± 17.2	762.6 ± 11.4	699.3 ± 50.4
α Cyclodextran	700.2 ± 39.4	824.8 ± 22.5*	701.2 ± 14.0	898.8 ± 55.0**	—
Leukocytes (10^3/μL)					
Corn oil	7.46 ± 0.60	—	7.54 ± 0.48	8.02 ± 0.39	6.70 ± 0.62
α-Cyclodextran	8.44 ± 0.49	8.24 ± 0.57	7.96 ± 0.49	8.52 ± 0.61	—
Segmented neutrophils (10^3/μL)					
Corn oil	1.38 ± 0.11	—	1.43 ± 0.10	1.74 ± 0.16	1.52 ± 0.13
α Cyclodextran	2.19 ± 0.26	2.04 ± 0.29	1.54 ± 0.20	2.16 ± 0.35	—
Lymphocytes (10^3/μL)					
Corn oil	5.28 ± 0.51	—	5.27 ± 0.38	5.27 ± 0.31	4.19 ± 0.52
α Cyclodextran	5.16 ± 0.39	5.19 ± 0.25	5.48 ± 0.40	5.39 ± 0.48	—
Activated lymphocytes (10^3/μL)					
Corn oil	0.44 ± 0.05	—	0.49 ± 0.03	0.58 ± 0.10	0.53 ± 0.06
α Cyclodextran	0.56 ± 0.05	0.51 ± 0.07	0.54 ± 0.11	0.58 ± 0.04	—
Monocytes (10^3/μL)					
Corn oil	0.26 ± 0.04	—	0.27 ± 0.03	0.31 ± 0.02	0.29 ± 0.04
α Cyclodextran	0.38 ± 0.01	0.33 ± 0.05	0.28 ± 0.03*	0.27 ± 0.02*	—
Basophils (10^3/μL)					
Corn oil	0.04 ± 0.01	—	0.03 ± 0.00	0.05 ± 0.02	0.05 ± 0.01
α Cyclodextran	0.04 ± 0.01	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.00	—
Eosinophils (10^3/μL)					
Corn oil	0.06 ± 0.01	—	0.07 ± 0.01	0.06 ± 0.01	0.09 ± 0.02
α Cyclodextran	0.12 ± 0.01	0.10 ± 0.02	0.09 ± 0.01	0.09 ± 0.01	—

TABLE B1 Hematology Data for F344/N Rats in the 14-Day Gavage Study of *p*-Chloro-α,α,α-trifluorotoluene (continued)

[1] Data are presented as mean ± standard error
[2] Not tested at this dose level
[3] n=4
* Statistically significantly different (P≤0 05) from the control group by Dunn's or Shirley's test
** Statistically significantly different (P≤0 01) from the control group by Dunn's or Shirley's test

TABLE B2 Clinical Chemistry Data for F344/N Rats in the 14-Day Gavage Study of *p*-Chloro-α,α,α-trifluorotoluene[1]

Analysis	0 mg/kg	10 mg/kg	50 mg/kg	400 mg/kg	1000 mg/kg
MALE					
n	5	5	5	5	5
Albumin (g/dL)					
Corn oil	3 8 ± 0 1	—[2]	3 9 ± 0 1	3 9 ± 0 1	3 9 ± 0 1
α-Cyclodextran	3 8 ± 0 1	3 8 ± 0 0	3 9 ± 0 0	4 0 ± 0 1	—
Alkaline phosphatase (IU/L)					
Corn oil	353 ± 11	—	370 ± 20	347 ± 11	414 ± 3*
α-Cyclodextran	326 ± 9	331 ± 5	344 ± 5	327 ± 17	—
Alanine aminotransferase (IU/L)					
Corn oil	31 ± 1	—	29 ± 2	28 ± 1	34 ± 2
α-Cyclodextran	30 ± 1	29 ± 1	31 ± 1	29 ± 2	—
Bile acids (μmol/L)					
Corn oil	24 26 ± 2 98	—	24 84 ± 3 34	26 48 ± 1 22	54 00 ± 11 87**
α-Cyclodextran	29 42 ± 5 99	32 86 ± 5 56	23 46 ± 3 63	24 84 ± 3 44	—
Chloride (mEq/L)					
Corn oil	103 ± 2	—	103 ± 2	102 ± 1	101 ± 2
α-Cyclodextran	103 ± 2	104 ± 2	103 ± 2	103 ± 2	—
Cholesterol (IU/L)					
Corn oil	57 0 ± 1 1	—	61 6 ± 1 6	86 2 ± 2 4**	133 6 ± 3 3**
α-Cyclodextran	62 6 ± 1 3	64 2 ± 2 0	73 4 ± 4 4*	85 8 ± 2 2**	—
Creatine kinase (IU/L)					
Corn oil	37 ± 7	—	29 ± 1	36 ± 3[3]	30 ± 2
α-Cyclodextran	32 ± 3	36 ± 5	31 ± 2	33 ± 5[3]	—
Creatinine (mg/dL)					
Corn oil	0 84 ± 0 02	—	0 90 ± 0 03	0 90 ± 0 03	0 94 ± 0 02*
α Cyclodextran	0 88 ± 0 02	0 82 ± 0 02	0 90 ± 0 00	0 86 ± 0 02	—
5-Nucleotidase (IU/L)					
Corn oil	23 00 ± 0 89	—	25 40 ± 0 87	26 00 ± 0 71*	37 20 ± 2 44**
α-Cyclodextran	23 00 ± 0 32	24 00 ± 0 45	25 40 ± 0 75**	26 40 ± 1 29*	—
Potassium (mEq/L)					
Corn oil	6 ± 0	—	6 ± 0	6 ± 0	7 ± 0
α-Cyclodextran	6 ± 0	6 ± 0	6 ± 0	6 ± 0	—
Sorbitol dehydrogenase (IU/L)					
Corn oil	14 ± 1	—	14 ± 1	16 ± 1	19 ± 1**
α Cyclodextran	14 ± 0	12 ± 1	14 ± 1	16 ± 1	—
Serum glucose (mg/dL)					
Corn oil	143 ± 6	—	138 ± 5	125 ± 6	143 ± 7
α Cyclodextran	142 ± 3	150 ± 10	141 ± 7	124 ± 4	—
Sodium (mEq/L)					
Corn oil	158 ± 2	—	160 ± 2	158 ± 3	156 ± 3
α Cyclodextran	157 ± 3	156 ± 2	158 ± 2	156 ± 3	—
Total protein (g/dL)					
Corn oil	8 1 ± 0 1	—	8 4 ± 0 2	8 7 ± 0 2*	9 1 ± 0 2**
α-Cyclodextran	8 4 ± 0 2	8 1 ± 0 1	8 6 ± 0 1	8 7 ± 0 2	—
Triglyceride (mg/dL)					
Corn oil	143 ± 20	—	157 ± 3	167 ± 18	220 ± 30
α-Cyclodextran	199 ± 17	205 ± 22	183 ± 21	164 ± 8	—
Urea nitrogen (mg/dL)					
Corn oil	19 0 ± 0 7	—	19 2 ± 0 6	19 2 ± 0 7	21 0 ± 0 6
α-Cyclodextran	22 6 ± 1 2	23 8 ± 0 7	22 6 ± 0 9	22 2 ± 0 9	—

TABLE B2 Clinical Chemistry Data for F344/N Rats in the 14-Day Gavage Study of *p*-Chloro-α,α,α-trifluorotoluene (continued)

Analysis	0 mg/kg	10 mg/kg	50 mg/kg	400 mg/kg	1000 mg/kg
FEMALE					
n	5	5	5	5	4
Albumin (g/dL)					
Corn oil	3 8 ± 0 1	—	3 9 ± 0 1	3 9 ± 0 1	4 2 ± 0 2*
α-Cyclodextran	3 9 ± 0 1	3 8 ± 0 1	3 8 ± 0 1	3 8 ± 0 1	—
Alkaline phosphatase (IU/L)					
Corn oil	341 ± 29	—	320 ± 24	320 ± 4	331 ± 9
α-Cyclodextran	253 ± 21	247 ± 11	294 ± 12	229 ± 12	—
Alanine aminotransferase (IU/L)					
Corn oil	32 ± 3	—	29 ± 0	32 ± 2	29 ± 3
α-Cyclodextran	33 ± 2	31 ± 2	33 ± 1	24 ± 1**	—
Bile acids (μmol/L)					
Corn oil	42 26 ± 9 03	—	34 94 ± 5 95	56 06 ± 12 28	78 87 ± 17 46
α-Cyclodextran	52 78 ± 12 05	54 18 ± 18 86	66 50 ± 7 36	59 85 ± 1 61[3]	—
Chloride (mEq/L)					
Corn oil	106 ± 2	—	106 ± 2	104 ± 2	104 ± 2
α-Cyclodextran	107 ± 2	106 ± 2	105 ± 2	105 ± 2	—
Cholesterol (IU/L)					
Corn oil	77 4 ± 3 8	—	86 4 ± 2 9	122 0 ± 6 4**	169 5 ± 11 8**
α-Cyclodextran	89 2 ± 4 2	87 4 ± 4 3	95 4 ± 5 3	120 6 ± 2 8**	—
Creatine kinase (IU/L)					
Corn oil	21 ± 3[3]	—	29 ± 1	29 ± 4	39 ± 10
α-Cyclodextran	37 ± 12[3]	22 ± 2	34 ± 10	32 ± 10	—
Creatinine (mg/dL)					
Corn oil	0 82 ± 0 02	—	0 82 ± 0 04	0 84 ± 0 02	0 88 ± 0 03
α Cyclodextran	0 84 ± 0 05	0 86 ± 0 02	0 80 ± 0 03	0 84 ± 0 02	—
5-Nucleotidase (IU/L)					
Corn oil	25 20 ± 1 02	—	27 00 ± 1 45	30 40 ± 0 98*	36 75 ± 3 47**
α Cyclodextran	25 80 ± 0 97	25 60 ± 1 25	26 60 ± 0 40	26 60 ± 0 68	—
Potassium (mEq/L)					
Corn oil	5 ± 0	—	6 ± 0	5 ± 0	6 ± 0
α-Cyclodextran	6 ± 0	6 ± 0	5 ± 0	6 ± 0	—
Sorbitol dehydrogenase (IU/L)					
Corn oil	14 ± 1	—	16 ± 1	16 ± 1	16 ± 1
α-Cyclodextran	15 ± 1	14 ± 1	15 ± 1	16 ± 1	—
Serum glucose (mg/dL)					
Corn oil	129 ± 4	—	130 ± 5	128 ± 4	134 ± 7
α-Cyclodextran	140 ± 15	144 ± 11	128 ± 7	148 ± 3	—
Sodium (mEq/L)					
Corn oil	155 ± 3	—	155 ± 3	154 ± 3	155 ± 3
α-Cyclodextran	157 ± 4	156 ± 3	155 ± 3	156 ± 3	—
Total protein (g/dL)					
Corn oil	7 5 ± 0 2	—	7 9 ± 0 2	8 5 ± 0 3*	8 8 ± 0 4**
α Cyclodextran	8 0 ± 0 2	7 8 ± 0 2	7 8 ± 0 1	7 9 ± 0 1	—
Triglyceride (mg/dL)					
Corn oil	111 ± 10	—	115 ± 17	127 ± 5	190 ± 39*
α-Cyclodextran	125 ± 9	107 ± 6	99 ± 6*	101 ± 8	—
Urea nitrogen (mg/dL)					
Corn oil	18 8 ± 1 3	—	19 6 ± 1 2	18 8 ± 0 9	23 0 ± 1 2*
α-Cyclodextran	23 4 ± 0 4	21 8 ± 1 5	23 4 ± 0 9	20 6 ± 0 2**	—

[1] Data are presented as mean ± standard error
[2] Not tested at this dose level
[3] n=4
* Statistically significantly different (P≤0 05) from the control group by Dunn's or Shirley's test
** Statistically significantly different (P≤0 01) from the control group by Dunn's or Shirley's test

TABLE B3 Clinical Chemistry Data for B6C3F$_1$ Mice in the 14-Day Gavage Study of *p*-Chloro-α,α,α-trifluorotoluene[1]

Analysis	0 mg/kg	10 mg/kg	50 mg/kg	400 mg/kg	1000 mg/kg
MALE					
n	5	5	5	5	5
Albumin (g/dL)					
Corn oil	3.4 ± 0.2[2]	3.2 ± 0.2	3.3 ± 0.2	3.4 ± 0.0	3.4 ± 0.2
α-Cyclodextran	3.5 ± 0.1	3.8 ± 0.0**[2]	3.5 ± 0.1	3.5 ± 0.1	–[3]
Alkaline phosphatase (IU/L)					
Corn oil	189 ± 31[2]	155 ± 22	154 ± 27	191 ± 13	138 ± 27
α-Cyclodextran	176 ± 20	186 ± 7[2]	191 ± 11	183 ± 19	–
Alanine aminotransferase (IU/L)					
Corn oil	23 ± 3[2]	24 ± 4	19 ± 2	30 ± 1	40 ± 5*
α-Cyclodextran	41 ± 5[2]	63 ± 11[2]	29 ± 3	38 ± 3	–
Bile acids (μmol/L)					
Corn oil	14.10 ± 2.40[2]	12.38 ± 1.92	11.20 ± 1.72[2]	16.88 ± 2.42	21.34 ± 1.37
α-Cyclodextran	13.78 ± 0.73[2]	10.30 ± 2.26[2]	12.52 ± 0.68	18.00 ± 1.64	–
Cholesterol (IU/L)					
Corn oil	132.5 ± 10.5[2]	117.8 ± 11.7	141.8 ± 19.6	154.0 ± 8.8	244.0 ± 21.3*
α-Cyclodextran	127.4 ± 4.3	140.8 ± 4.2[2]	133.8 ± 6.0	160.4 ± 7.0**	–
Creatine kinase (IU/L)					
Corn oil	135 ± 24[2]	158 ± 31	134 ± 25	174 ± 38	157 ± 47
α-Cyclodextran	167 ± 22	154 ± 16[4]	210 ± 44[2]	207 ± 56[2]	–
Creatinine (mg/dL)					
Corn oil	0.70 ± 0.04[2]	0.64 ± 0.02	0.64 ± 0.02	0.70 ± 0.05	0.68 ± 0.04
α-Cyclodextran	0.66 ± 0.02	0.70 ± 0.00[2]	0.62 ± 0.02	0.72 ± 0.02	–
5-Nucleotidase (IU/L)					
Corn oil	18.25 ± 0.85[2]	19.40 ± 1.29	18.20 ± 0.66	20.20 ± 0.37	48.60 ± 6.11**
α Cyclodextran	20.20 ± 1.32	19.50 ± 1.26[2]	17.00 ± 0.32	24.80 ± 1.77	–
Sorbitol dehydrogenase (IU/L)					
Corn oil	30 ± 3[2]	27 ± 2	29 ± 2	30 ± 1	41 ± 3
α Cyclodextran	36 ± 1[2]	37 ± 2[2]	33 ± 1	32 ± 1	–
Serum glucose (mg/dL)					
Corn oil	179 ± 9[2]	170 ± 12	165 ± 18	204 ± 7	179 ± 17
α-Cyclodextran	177 ± 12	197 ± 11[2]	187 ± 15	215 ± 10*	–
Total protein (g/dL)					
Corn oil	6.7 ± 0.2[2]	6.6 ± 0.2	7.0 ± 0.2	6.9 ± 0.2	7.9 ± 0.3*
α-Cyclodextran	6.8 ± 0.1	7.2 ± 0.2[2]	6.9 ± 0.1	7.0 ± 0.2	–
Triglyceride (mg/dL)					
Corn oil	131 ± 30[2]	105 ± 10	113 ± 16	127 ± 16	137 ± 25
α-Cyclodextran	116 ± 4	146 ± 25[2]	170 ± 8**	177 ± 13**	–
Urea nitrogen (mg/dL)					
Corn oil	23.5 ± 1.7[2]	22.0 ± 0.7	20.6 ± 1.6	24.0 ± 1.1	19.8 ± 1.5
α-Cyclodextran	21.2 ± 1.7	20.3 ± 1.8[2]	23.4 ± 0.9	22.4 ± 1.6	–

TABLE B3 Clinical Chemistry Data for B6C3F$_1$ Mice in the 14-Day Gavage Study of *p*-Chloro-α,α,α-trifluorotoluene (continued)

Analysis	0 mg/kg	10 mg/kg	50 mg/kg	400 mg/kg	1000 mg/kg
FEMALE					
n	5	5	5	5	5
Albumin (g/dL)					
Corn oil	3 7 ± 0 1	3 6 ± 0 1	3 8 ± 0 1	3 6 ± 0 1	3 6 ± 0 1
α-Cyclodextran	3 8 ± 0 0	3 9 ± 0 1[4]	3 8 ± 0 1[2]	3 8 ± 0 1	–
Alkaline phosphatase (IU/L)					
Corn oil	357 ± 13	327 ± 9	354 ± 19	288 ± 48	303 ± 15*
α-Cyclodextran	324 ± 13	319 ± 5[4]	339 ± 17[2]	329 ± 22	–
Alanine aminotransferase (IU/L)					
Corn oil	26 ± 2	24 ± 1	24 ± 1	24 ± 1	33 ± 6
α-Cyclodextran	28 ± 2	30 ± 1[4]	30 ± 2[2]	28 ± 2	–
Bile acids (μmol/L)					
Corn oil	19 76 ± 0 64	21 24 ± 1 96	19 02 ± 1 59	20 38 ± 2 55	23 34 ± 1 99
α-Cyclodextran	17 94 ± 1 59	18 13 ± 1 77[4]	16 40 ± 1 19[2]	19 12 ± 0 23	–
Cholesterol (IU/L)					
Corn oil	118 4 ± 4 9	110 0 ± 4 9	113 2 ± 3 4	141 4 ± 6 8	194 8 ± 9 3**
α Cyclodextran	112 0 ± 4 7	111 0 ± 8 7[4]	115 3 ± 10 8[2]	152 6 ± 5 8*	–
Creatine kinase (IU/L)					
Corn oil	168 ± 46	193 ± 58	80 ± 30	149 ± 25	157 ± 41
α Cyclodextran	117 ± 24	195 ± 27[4]	247 ± 63[2]	156 ± 42	–
Creatinine (mg/dL)					
Corn oil	0 64 ± 0 02	0 66 ± 0 04	0 68 ± 0 04	0 70 ± 0 03	0 76 ± 0 04*
α Cyclodextran	0 68 ± 0 04	0 70 ± 0 06[4]	0 65 ± 0 03[2]	0 66 ± 0 02	–
5 Nucleotidase (IU/L)					
Corn oil	88 00 ± 3 22	88 00 ± 2 45	104 40 ± 7 90	113 80 ± 8 22	227 60 ± 10 93**
α Cyclodextran	104 80 ± 5 47	98 00 ± 7 57[4]	94 50 ± 7 44[2]	136 20 ± 4 90*	–
Sorbitol dehydrogenase (IU/L)					
Corn oil	27 ± 1	23 ± 2	28 ± 2	28 ± 1	36 ± 4
α Cyclodextran	28 ± 2	30 ± 3[4]	30 ± 1[2]	31 ± 2	–
Serum glucose (mg/dL)					
Corn oil	197 ± 14	224 ± 23	190 ± 20	193 ± 15	186 ± 13
α Cyclodextran	160 ± 8	165 ± 11[4]	176 ± 8[2]	172 ± 9	–
Total protein (g/dL)					
Corn oil	6 9 ± 0 2	6 6 ± 0 2	6 9 ± 0 2	7 0 ± 0 2	7 3 ± 0 2
α Cyclodextran	7 1 ± 0 1	7 3 ± 0 1[4]	7 1 ± 0 1[2]	7 4 ± 0 1	–
Triglyceride (mg/dL)					
Corn oil	135 ± 10	109 ± 11	149 ± 17	159 ± 22	240 ± 31*
α Cyclodextran	121 ± 14	139 ± 13[4]	139 ± 8[2]	206 ± 49	–
Urea nitrogen (mg/dL)					
Corn oil	20 8 ± 1 0	21 0 ± 1 1	27 4 ± 3 0	22 0 ± 0 9	22 6 ± 2 3
α Cyclodextran	27 2 ± 2 0	23 0 ± 1 5[4]	26 0 ± 3 1[2]	26 2 ± 2 9	–

[1] Data are presented as mean ± standard error
[2] n=4
[3] Not tested at this dose level
[4] n=3
* Statistically significantly different (P≤0 05) from the control group by Dunn s or Shirley s test
** Statistically significantly different (P≤0 01) from the control group by Dunn s or Shirley s test

APPENDIX C

Genetic Toxicology

Table C1 Mutagenicity of *p*-Chloro-α,α,α-trifluorotoluene in *Salmonella typhimurium*[1]

Strain	Dose (μg/plate)	Revertants/plate[2] - S9 Trial 1	- S9 Trial 2	+ Hamster S9 Trial 1	+ Hamster S9 Trial 2	+ Rat S9 Trial 1	+ Rat S9 Trial 2
TA100	0	111 ± 6 1	92 ± 12 6	99 ± 3 5	98 ± 6 7	111 ± 12 5	123 ± 4 9
	10	113 ± 5 5	90 ± 11 1	104 ± 2 9	96 ± 10 2	115 ± 6 3	101 ± 7 6
	33	105 ± 4 5	99 ± 7 6	106 ± 3 9	88 ± 9 7	101 ± 1 2	103 ± 11 4
	100	100 ± 5 2	103 ± 16 2	98 ± 3 8	106 ± 3 5	102 ± 4 2	88 ± 1 5
	333	110 ± 0 9	82 ± 4 5[3]	74 ± 4 6	101 ± 2 5	73 ± 2 3	72 ± 10 7[3]
	1000	Toxic	Toxic	64 ± 6 2[3]	92 ± 8 7	64 ± 4 7[3]	67 ± 1 2[3]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control[4]		483 ± 7 2	416 ± 11 3	1119 ± 119 8	2115 ± 14 6	1075 ± 30 0	549 ± 71 3
TA1535	0	32 ± 2 3	20 ± 4 4	12 ± 2 2	10 ± 1 7	14 ± 0 6	8 ± 1 7
	10	38 ± 0 6	16 ± 1 2	16 ± 1 5	15 ± 1 3	15 ± 0 7	12 ± 0 0
	33	33 ± 4 3	14 ± 1 5	12 ± 1 5	12 ± 2 9	16 ± 2 7	9 ± 1 3
	100	36 ± 3 7	15 ± 3 0	15 ± 2 8	10 ± 1 8	12 ± 2 2	14 ± 1 5
	333	28 ± 2 3[3]	13 ± 3 4[3]	8 ± 0 9	9 ± 2 6	11 ± 3 4	8 ± 2 3[3]
	1000	Toxic	8 ± 0 0[3]	7 ± 1 5[3]	11 ± 1 3	11 ± 2 7[3]	11 ± 2 3[3]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		412 ± 9 4	346 ± 14 4	257 ± 13 8	266 ± 9 5	314 ± 14 9	167 ± 4 9
TA1537	0	16 ± 3 8	5 ± 1 7	16 ± 2 1	8 ± 1 3	16 ± 1 5	6 ± 1 2
	10	10 ± 2 7	7 ± 1 9	23 ± 1 3	6 ± 0 3	19 ± 0 6	6 ± 0 9
	33	11 ± 1 2	3 ± 0 6	21 ± 1 8	6 ± 1 2	6 ± 0 3	7 ± 0 3
	100	9 ± 1 5	4 ± 1 3	19 ± 0 9	6 ± 1 2	5 ± 0 9	6 ± 1 2
	333	8 ± 0 3	4 ± 0 6	15 ± 0 9	6 ± 1 8	0 ± 3 8	5 ± 0 6
	1000	3 ± 2 5[3]	Toxic	15 ± 3 3[3]	3 ± 1 5[3]	7 ± 1 2[3]	1 ± 1 0[3]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		329 ± 159 1	847 ± 54 3	459 ± 52 4	411 ± 10 3	495 ± 52 6	239 ± 24 6
TA98	0	29 ± 0 9	34 ± 4 5	47 ± 7 6	33 ± 2 3	40 ± 3 3	43 ± 1 0
	10	31 ± 7 1	29 ± 1 8	52 ± 4 4	37 ± 2 0	53 ± 1 2	39 ± 3 3
	33	21 ± 1 5	29 ± 0 7	50 ± 8 7	35 ± 3 2	44 ± 5 7	35 ± 3 8
	100	2 ± 3 5	25 ± 6 6	48 ± 5 3	33 ± 4 2	32 ± 2 6	38 ± 3 6
	333	2 ± 2 4	8 ± 1 3[3]	28 ± 5 1	33 ± 3 4	22 ± 2 8	30 ± 5 5[3]
	1000	Toxic	Toxic	35 ± 2 1[3]	25 ± 5 5	22 ± 3 5[3]	27 ± 2 4[3]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		691 ± 10 1	671 ± 57 5	570 ± 57 5	1271 ± 7 8	574 ± 22 3	365 ± 22 9

[1] Study performed at SRI International The detailed protocol and these data are presented in Haworth *et al* (1983) Cells and study compound or solvent (dimethylsulfoxide) were incubated in the absence of exogenous metabolic activation (S9) or with Aroclor 1254 induced S9 from male Syrian hamster liver or male Sprague Dawley rat liver High dose was limited by toxicity or solubility but did not exceed 10 mg/plate 0 μg/plate dose is the solvent control

[2] Revertants are presented as mean ± the standard error from 3 plates

[3] Slight toxicity

[4] The positive controls in the absence of metabolic acitvation were 4 nitro *o*-phenylenediamine (TA98) sodium azide (TA100 and TA1535) and 9 aminoacridine (TA1537) The positive control for metabolic activation with all strains was 2 aminoanthracene