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Metabolism by Rats of 2-Dodecylcyclobutanone, a Radiolytic Compound Present in Irradiated Beef

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Alkylcyclobutanones (2-ACBs) are suspected cancer promoters and clastogens, which have raised concerns about the safety of irradiated foods. Currently there are few data on the metabolism of 2-ACBs, which makes it very important to study this aspect of 2-ACBs to evaluate their safety. The objectives of this experiment were to quantify 2-dodecylcyclobutanone (2-DCB; formed from palmitic acid) in the feces and adipose tissue of rats and to check for metabolites of 2-DCB in the urine. Six female Sprague−Dawley rats were administered 2-DCB (5 mg/day) in corn oil for 5 days via gavage. Six control rats did not receive 2-DCB. Feces and urine were collected daily, whereas adipose tissue was collected upon euthanasia. Hexane extracts of feces and adipose tissue were analyzed by gas chromatography−mass spectroscopy (GC-MS). Urine with and without added β-glucuronidase was monitored for glucuronide complexes by hexane extraction and GC-MS. The total amount of 2-DCB recovered in feces was 1.78 ± 0.63 mg at the end of 5 days, which represents between 3 and 11% of the total 2-DCB administered. The total amount recovered in the adipose tissue was 0.08 ± 0.01 mg, which was ≈0.33% of the total 2-DCB administered. No metabolites were recovered in any of the urine extracts. The results show that at most 11% of the 2-DCB was recovered unchanged in the feces and adipose tissue. This indicates that either most of 2-DCB is metabolized and rapidly eliminated from the body or stored at sites other than adipose tissue.

KEYWORDS: 2-Alkylcyclobutanone; 2-dodecylcyclobutanone; metabolism; irradiation; mass spectroscopy

INTRODUCTION

Alkylcyclobutanones are radiolytic products that are formed when triglycerides are subjected to ionizing radiation. These alkylcyclobutanones are cyclic compounds formed by the loss of an electron from the oxygen on the carbonyl of a fatty acid or triglyceride, followed by a rearrangement process to produce 2-alkylcyclobutanones (2-ACBs) specific to the parent fatty acid (*1*). Accordingly, when irradiated, the four major fatty acids present in most foods (palmitic, stearic, oleic, and linoleic acids) are converted to their corresponding cyclobutanones, namely, 2-dodecylcyclobutanone (2-DCB), 2-tetradecyl- and 2-tetradec-5′-enylcyclobutanone (2-TDCB), and 2-tetradeca-5′,8′-dienylcyclobutanone.

First discovered in 1972 in irradiated oils, 2-ACBs have been detected in a wide variety of irradiated lipid-containing foods (*2*−*5*). They are not formed in cooked or processed foods, which has made them useful as indicators of irradiation exposure. Studies conducted on γ-irradiated chicken, peanuts, perilla seeds, and pork (*6*−*9*) have shown that the concentration of alkylcyclobutanones increased linearly with irradiation dose. Experiments on irradiated ground beef have shown that 2-DCB can be used to estimate the absorbed dose of commercially irradiated samples (*5*, *10*).

The unique nature of 2-ACBs has generated considerable controversy with regard to their toxicity. Even though there are ample data on the occurrence of 2-ACBs in irradiated foods, there are limited data on their toxicity or metabolism. Some studies have shown that 2-ACBs are genotoxic (*11*), can cause DNA strand breaks (*12*), can weakly induce the formation of micronuclei in lymphoblasts after DNA strand breaks (*13*), and may be cancer promoters (*14*). Other studies of 2-ACBs investigating mutagenicity and chromosomal damage have failed to show any adverse effects (*15*−*18*). These contradictory data have been used to cast doubt on the safety of irradiated foods by some consumer organizations. The biggest hurdle in countering these claims has been the lack of literature on the toxicity and metabolism of 2-ACBs. There are more than enough data that show no adverse effects of irradiated foods (*19*) but very little data on the effects of pure 2-ACBs.

There is only one published study on the fate of 2-ACBs after consumption (*20*). Male Wistar rats were fed ≈1 mg of 2-tetradecyl- or 2-tetradecenylcyclobutanone in drinking water each day for 4 months. Although 2-ACBs are fat-soluble substances, the amount recovered in the adipose tissue was ≈100 000-fold less than the total quantity consumed. In addition,

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only 0.1−0.3% of the amount consumed daily was detected in fecal matter. This study provides evidence that these compounds do not accumulate in adipose tissue and may be metabolized and/or degraded in animals. The authors did not evaluate the formation of metabolites.

Elliott et al. (*21*) proposed a conversion of 2-DCB to γ-palmitolactone when it was injected into the body of a rabbit. Antibodies were raised against 11-(2′-oxocyclobutyl)undecanoic acid, a 2-substituted cyclobutanone with a terminal carboxyl group, to develop an enzyme-linked immunosorbent assay (ELISA) to detect 2-ACBs. The antibody had significant cross-reactivity not only with 2-DCB but also with its corresponding lactone. Elliott et al. (*21*) concluded that during or after conjugation or inoculation a significant portion of the 2-ACB was converted to the lactone. Hamilton et al. (*22*) observed the formation of γ-palmitolactone [5-dodecyldihydro-2(3*H*)-furanone] when 2-DCB was stored as a hexane solution stored for several months, a result that has been confirmed in our laboratory. Vajdi et al. (*23*) reported the formation of long-chain γ and δ lactones in beef irradiated at very high doses. An oxidative process was proposed to explain the formation of lactones from fatty acids and triglycerides. As these lactones could also be formed from the corresponding 2-ACB oxidation, it is possible that a similar process can occur in animal tissues.

The ring structure of the lactone is very similar to that of furan. Furan is a five-membered ring structure containing double bonds and an oxygen as part of the ring structure. In rats exposed to [^{14}C]furan for 24 h, almost 80% of the radioactivity was eliminated primarily through urine and expired air. Almost 26% of the administrated furan was expired as CO_2, whereas ≈14% was expired unchanged (*24*). Carbon dioxide was a major metabolite, indicating that the furan ring opened up during metabolism. It is possible that the 2-ACBs may follow a similar path in which the alkyl constituent may be cleaved from the ring followed by opening and oxidation of the lactone ring.

Another possibility is the conversion of the cyclobutanone moiety of the 2-ACBs to an alcohol or diol, similar to cyclohexanone. Cyclohexanone is a cyclic ketone containing six carbon atoms,which is similar to the cyclobutanone ring of 2-ACBs. In a study conducted by Mraz et al. (*25*), it was shown that in humans exposed to cyclohexanone, it was excreted via urine, with 1,2- and 1,4-cyclohexanediol being the major urinary metabolites. The alcohol metabolites were excreted as β-glucuronide complexes.

The fate of 2-DCB in rats after consumption has not yet been studied. Therefore, we were interested in finding out if 2-DCB could be recovered from the feces and adipose tissue of rats and if any urinary metabolites of 2-DCB could be identified. Thus, the objectives of this experiment were (1) to quantify 2-DCB in the feces and adipose tissue of rats and (2) to identify any urinary metabolites of 2-DCB such as an alcohol or a lactone.

MATERIALS AND METHODS

Chemicals and Reagents. Hexane, ether, and 2-DCB standard were purchased from Fisher Scientific (Pittsburgh, PA). Sodium acetate, β-glucuronidase, glycine, and bis(trimethylsilyl)trifluoroacetamide (BFTSA) were obtained from Sigma Aldrich (St. Louis, MO).

Preliminary Study. Four female Sprague−Dawley rats (200−250 g) were purchased from Harlan (Indianapolis, IN). Two rats were administered 5 mg of DCB/day dissolved in corn oil via gavage for 2 days. Control rats received corn oil only. The rats were housed in metabolism cages to facilitate the collection of urine and feces, which were collected daily. At the end of the experiment the rats were euthanized by carbon dioxide, after which the serum and adipose tissue were collected.

From the preliminary tests it was found that hexane extraction could be used to extract the feces. The 2-DCB could be extracted from the adipose tissue by supercritical fluid extraction (SFE). Simple hexane or ether extracts of urine did now show the presence of any metabolites. One hypothesis was that the ketone group of the 2-DCB may be converted to an alcohol (OH) group. Such groups can be detected by conversion to trimethylsilyl (TMS) derivatives. The alcohol might also be excreted as a glucuronide conjugate, which can be analyzed by hydrolysis with β-glucuronidase. Therefore, it was decided that TMS derivatization and incubation of urine with β-glucuronidase should be evaluated in the main study. The detailed procedures are described under the main study. The serum was analyzed by extraction with ether or hexane followed by gas chromatography−mass spectrometrey (GC-MS). The GC-MS chromatograms for the serum extracts did not show any peaks of interest.

Main Study. *Collection of Rat Feces, Urine, and Organs.* Twelve female Sprague−Dawley rats (200−250 g) were purchased from Harlan (Indianapolis, IN). Six of these were treatment rats, and six were control rats. The rats were housed in metabolism cages to facilitate the collection of urine and feces. Treatment rats were administered 1 mL of a 5 mg/mL solution of 2-DCB via gavage daily for 5 days. The 2-DCB was dissolved in corn oil prior to administration, and the control rats received 1 mL of corn oil only. The U.S. FDA Redbook 2000 (*26*) states the maximum volume of solution that can be given by gavage in one dose for rodents should not exceed 1 mL/100 g of body weight. A gavage volume of 1 mL was selected so that it would be well below the limit recommended by the FDA. Urine and feces were collected daily, and at the end of the experiment adipose tissue was collected after the rats had been euthanized by carbon dioxide.

Analysis of Feces. The feces from days 3−5 for each rat were pooled and homogenized by crushing. Between 2 and 3 g of feces was weighed and extracted with 10 mL of hexane three times. Each time the feces were shaken with 10 mL of hexane in a wrist action shaker, after which the extracts were filtered through Whatman filter paper no. 4. The filtrate was concentrated under nitrogen to 1 mL, and 1 μL was injected for GC-MS analysis. The feces were extracted two times, and each extract was injected into the GC twice to obtain a total of four values for the 2-DCB recovery.

Analysis of Adipose Tissue. The adipose tissues from each of the treatment and control rats were pooled. The adipose tissue was homogenized with wet support in the ratio 1:2 and extracted by SFE. An ISCO-Suprex prepmaster GA (Teledyne Isco, Inc., Lincoln, NE) fat analyzer was used for the SFE procedure. A 5 mL SFE cartridge (Teledyne Isco, Inc.) was loaded with sand, Florisil, and ≈1.5 g of the adipose tissue−wet support mixture prior to being placed in the extractor. The sand protects the seals of the extraction cartridge, whereas the Florisil serves to trap the fat. The adipose tissue was extracted three times.

Extraction was carried out under the following conditions: pressure, 340 atm; temperature, 75 °C; 5 min static and 20 min dynamic with a flow rate of CO_2 of 1 mL/min. The final extract volume was 25 μL, and 1 μL was used for GC-MS analysis.

Urine Analysis. *TMS Derivative.* Urine from all 5 days was pooled for each rat. Two milliliters of urine was evaporated to dryness with a rotary evaporator. To this were added 100 μL of pyridine and 100 μL of BFTSA silylating agent, and the extracts were transferred to a reaction vial. The vials were heated at 65 °C for 30 min and cooled, and 1 μL was used for GC-MS analysis. Palmitic acid (0.5 mg) and 2-DCB (0.5 mg) were also treated with BFTSA similarly to check for TMS derivatives.

β-Glucuronidase Assay. One milliliter of urine was adjusted to pH 6.8 in a test tube, and 0.7 mL of 100 mM sodium acetate (pH 5.0) was added to it. After equilibration at 37 °C, ≈1000 units of β-glucuronidase was added to the test tube. Two aliquots from each rat were used. Urine without added β-glucuronidase was also analyzed. The urine was incubated overnight (∼18 h), and the aliquots were extracted with 5 mL of hexane. The hexane extracts were concentrated to 1 mL, and 1 μL was injected for GC-MS analysis. The activity of β-glucuronidase

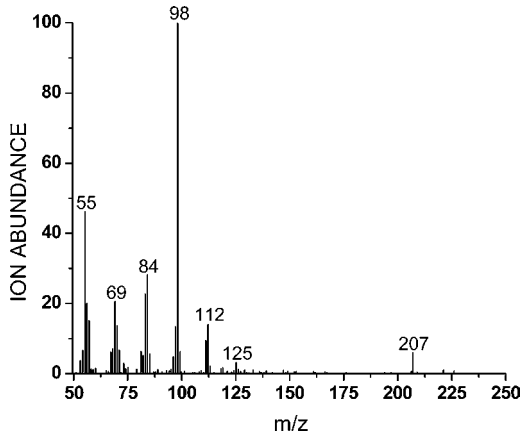

Figure 1. Mass spectrum of 2-DCB.

Table 1. Amount of 2-DCB Recovered in the Feces from Day 3 to Day 5 and Percent of the Total Amount of 2-DCB Administered (25 mg) (Total Amount Was Averaged from Duplicate Extracts and Assays)

rat	total amount of 2-DCB in feces (mg)	% of total 2-DCB
1	1.99	7.97
2	0.96	3.84
3	2.04	8.17
4	1.75	6.98
5	1.20	4.81
6	2.71	10.85
av	1.78 ± 0.63	7.10 ± 2.52

was checked according to the method obtained from Sigma-Aldrich (*27*). The average activity of β-glucuronidase was 547 units/mL.

GC-MS Analysis. GC-MS was performed with a HP 5890 mass spectrometer (Agilent Technologies, Palo Alto, CA) fitted with a HP-5 MS column (cross-linked 5% Ph Me siloxane, 30 m × 0.22 mm × 0.25 μm film thickness) and a HP MSD 5970 detector (Agilent Technologies). The flow rate for the helium carrier gas was 1 mL/min. The GC temperature program was as follows: injector temperature, 250 °C; initial temperature, 55 °C; hold, 0.5 min; ramp at 20 °C/min; final temperature, 200 °C; hold, 1 min; ramp 15 °C/min; final temperature, 270 °C; hold, 1 min. The transfer line and ion source were held at 280 °C throughout the runs. Standard solutions of 10, 25, 50, 100, 250, and 500 ppm in hexane were used to calibrate the standard curve for 2-DCB. The mass spectra were scanned between *m*/*z* 50 and 550. The compounds were identified by comparing retention times and the ion ratios of the full-scan mass spectrum with the standards. The ratios of ions *m*/*z* 98, 112, and 207 were used to confirm the presence of 2-DCB, and the concentration in the sample was determined from the standard curve. The mass spectrum of 2-DCB is shown in **Figure 1**.

RESULTS AND DISCUSSION

The average amount of 2-DCB recovered from the feces was 1.78 ± 0.63 mg (**Table 1**). Among the six treatment rats, this represents between 3 and 11% of the total amount given to the rats. The total amount of 2-DCB recovered from adipose tissue was 0.08 ± 0.01 mg, which was ≈0.33% of the total 2-DCB administered. This indicates that most of the 2-DCB is metabolized and excreted or stored in tissues other than adipose. **Figures 2** and **3** show the GC-MS chromatograms of the feces and adipose tissue extracts.

No metabolites could be identified in any of the urine extracts derivatized with the TMS. This suggests that the ketone group of 2-DCB may not be converted to the OH group representing a cylcobutanol derivative. The palmitic acid was converted to its TMS derivitive, but 2-DCB remained unchanged. Incubation with β-glucuronidase (bovine liver, type B-10) did not show any unique metabolites. There are several types of glucuronidases from various sources, each with slightly different specificities. It is possible that this enzyme was not able to hydrolyze any 2-DCB metabolites that may have been formed. It is possible that the 2-DCB is completely metabolized and converted to some, as yet, unidentified compound or compounds. If the cyclobutanone ring were to open up, then the molecule is very similar to palmitic acid and could be metabolized

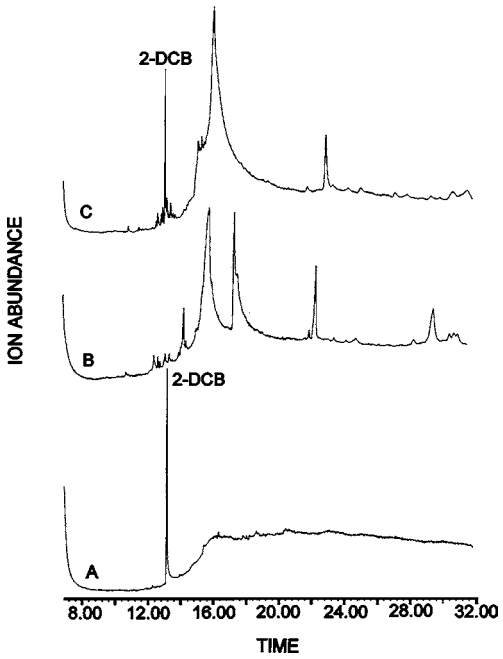

Figure 2. Total ion chromatogram (GC-MS) showing the presence of 2-DCB in the feces of rats: (**A**) 2-DCB standard; (**B**) control feces; (**C**) treatment feces.

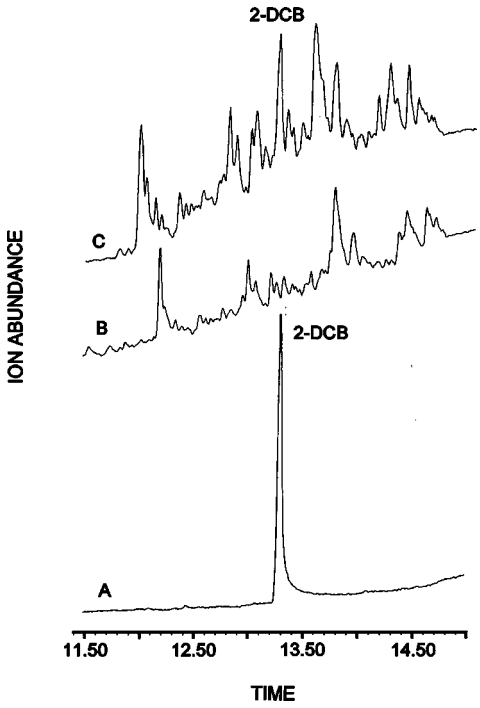

Figure 3. Total ion chromatograms (GC-MS) showing the presence of 2-DCB in the adipose tissue of rats: (**A**) 2-DCB standard; (**B**) control adipose tissue; (**C**) treatment adipose tissue.

similarly. If the molecule cleaves into various hydrocarbons, then it is possible that the end product of 2-DCB metabolism is CO_2 (*28*). These results indicated the 2-DCB might be cleared from the body fairly easily, and there might not be any concerns with bioaccumulation as in the case of DDT. Even at such a high dose, at most 11% of it passed through the colon unchanged.

The presence of 2-ACBs in the colon was regarded as a cause for concern by Horvatovich et al. (*20*) because of the adverse effects of 2-DCB on colon cells observed by Knoll et al. (*12*). The amount of 2-DCB found in commercial ground beef ground beef patties ranged from 0.03 to 0.05 ppm (*5*, *10*). For a patty weight of ≈115 g this amounts to ≈3.5−5.8 μg per patty, ≈30000−50000-fold less than the effective concentration used by Knoll et al. (*12*). The total exposure to 2-DCB by eating irradiated beef and poultry is estimated to be ≈0.19 μg per kg of body weight per day as calculated from a study conducted by Horvatovich et al. (*29*) and assuming the mean intake of poultry (62.1 g/day) and beef (23.2 g/day) (*30*). Therefore, a 70 kg adult would ingest ≈63 μg of 2-DCB/day, which is ≈2600-fold less than the effective concentration *in vitro*. Thus, the amount that would pass through the colon would be so small that any potential risk of 2-DCB would be minimal. Knoll et al. (*12*) state in their study that the amount found in the ground beef patties is "possibly too low to have a significant impact on human health."

An important point to be noted is that palmitic acid, from which 2-DCB is formed, is a strong inducer of oxidative DNA damage, DNA strand breaks, cell membrane damage, necrosis, and apoptosis in human and rodent cells *in vitro* at concentrations ranging from 50 to 200 mM (*31*, *32*). Other fatty acids such as linoleic acid (100 mM), arachidonic acid (100 mM), and thermally oxidized dietary oils cause cell membrane damage and chromosome fragmentation in human and rodent cells in vitro (*31*, *33*). Thus, the effects of 2-DCB on human primary cells and cells isolated from preoneoplastic lesions noted by Knoll et al. (*12*) appear to be similar to those caused by the palmitic acid parent molecule. Furthermore, the amount of palmitic acid in a cooked 110 g beef patty containing 20% fat is ≈3.8 g as listed in the USDA National Nutrient Database for Standard Reference (*34*). Thus, a single hamburger contains significantly more palmitic acid than the total estimated daily intake of 2-DCB. Given the nonmutagenic nature of 2-DCB, and the weak, clastogenic effect on human and rodent cells, it is difficult to perceive that consumption of irradiated foods containing 2-DCB will significantly increase the risk to human health.

At the same time, as there are so few data available on the effects of 2-ACBs, it would be advisable to collect more knowledge on the toxicological and metabolic properties of 2-ACBs. It is important to quantify a possible risk from 2-ACB consumption, however small it may be.

To fully placate public concerns about the consumption of irradiated foods, determining the fate of the 2-ACBs in the body is of great importance. If the 2-ACBs are metabolized rapidly, without adverse effects, and produce no harmful metabolites, public acceptance of irradiated foods should increase. As of now it is not easy for the consumers to make an informed decision about the safety of 2-ACBs.

ABBREVIATIONS USED

2-ACB, alkylcyclobutanone; 2-DCB, 2-dodecylcyclobutanone; BFTSA, bis(trimethylsilyl)trifluoroacetamide; SFE, supercritical fluid extraction; GC-MS, gas chromatography−mass spectroscopy.

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