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Determination of Tetrachlorodibenzo-p-dioxin (TCDD) in Human Adipose Tissue

PURPOSE: The Veterans Administration has undertaken a study to ascertain whether TCDD can be detected and measured in the body fat of human volunteers who believe that they were exposed to the defoliant Orange in Viet Nam and in the fat of other volunteers who have had no known contact with Orange. Some lots of the defoliant contain small amounts of TCDD as a contaminant.

BACKGROUND: A wide variety of late adverse effects have been said to occur years after exposure to the defoliant mixture called "Orange." In most cases involving Vietnam veterans it is difficult or impossible to document an individual's exposure to the defoliant mixture and it is rarely, if ever, possible to determine whether a person was exposed to the contaminant TCDD which varies in an undetermined manner from lot to lot. This contaminant is the most toxic constituent of the defoliant as judged by its acute toxicity in various animal species.

The body concentrates in its adipose tissue any TCDD it retains and some lower animals are known to retain it there for considerable periods. It has been suggested that individuals may sequester TCDD in their body fat and release it in toxic quantities when fat is suddenly decreased in amount even years later.

One individual who had been intensively exposed to TCDD in an industrial accident was found to have 0.04 to 1.04 parts per billion of thesubstance in varius organs and 1.84 parts per billion in body fat at autopsy several months after exposure. (Reggiani, reported at the 20th Congress of the European Society of Toxicology, June 25-28, 1978) It is unlikely that such high concentrations would persist in individuals exposed less intensively ten or more years earlier in Viet Nam. Since assay methods are being developed to identify and measure TCDD at concentrations of a few parts per trillion, the VA decided to determine whether such an experimental assay could detect the compound in human adipose tissue long after possible exposure.

Although the test was to be conducted on Viet Nam veterans who attribute adverse effects to their prior exposure, it was recognized that discovery of TCDD in their body fat could not prove that any defect, disease or disability is caused by the substance. Since TCDD occurs in defoliants or herbicides that have been used since the Viet Nam conflict, its presence in the fat of any one individual would not even prove conclusively that exposure occurred in Viet Nam. Detection of TCDD in his fat, would indicate only that a man had prior contact with the substance.

Relatively large samples of body fat would increase the likelihood of finding very low concentrations and the experimental nature of the assay techniques made adequate samples even more desirable. For these reasons, surgeons obtained the fat samples by biopsy.

METHODS: Fat Samples. Twenty Vietnam veterans who had conditions that they considered due to exposure to the defoliant Orange volunteered to have an open biopsy of subcutaneous adipose tissue from the anterior abdominal wall. Three Air Force officers who had repeated and relatively recent documented contact with Orange also volunteered. Eleven "control" veterans without known exposure had samples of subcutaneous fat removed during operations performed for other reasons, including herniorrhaphy, ureterolithotomy, and abdomino-peritoneal resection of a rectal adenocarcinoma. This control group matched the exposed group in age range, sex and general geographic location. All volunteers, both "control" and exposed, gave written informed consent.

The fat removed at biopsy weighted 3.8 grams or more except for one control sample of 1.4 grams; most specimens weighed 7 grams or more. Samples were placed in corked glass containers that had been rinsed with reagent grade acetone to remove interfering substances. The tissue was promptly chilled and kept refrigerated until assayed.

The assays, including the sample extraction, were performed by Michael L. Gross, Ph.D., at the Midwest Center for Mass Spectrometry. The National Science Foundation supports this Center.

Sample Extraction. In the assay laboratory, a 1 to 10 gram portion of the fat specimen was weighed and spiked with a known amount of TCDD containing non-radioactive but unnatural ^{37}Cl that the assay can distinguish from the natural compound. The sample was completely hydrolyzed by saponification in 15 ml of ethanol and 30 ml of 40% aqueous KOH in a reflux apparatus for 60 minutes without stirring. All solvents were of the highest grade and suitable for residue analysis.

The solution, diluted with 20 ml of ethanol and 40 ml of water, was extracted four times with nanograde hexane. The first extraction was with 25 ml of hexane, using vigorous shaking for one minute. The aqueous layers was separated and extracted three more times with 15 ml of hexane. The four hexane extracts were combined and washed with 10 ml of water to remove excess base followed by washes with 10 ml of concentrated sulfuric acid repeated four times or until both layers were clear. After a final washing with 10 ml of water, the hexane layer was concentrated to approximately 1 ml under a stream of dry nitrogen.

The first of three chromatography steps used unactivated silica gel as a 5 cm column in a disposable pipet plugged with glass wool. The silica was capped with 0.25 cm of anhydrous sodium sulfate to remove water and was wetted with hexane before the sample, dissolved in 1 ml of hexane was transferred to the column. The sample container was rinsed with a second milliliter that was subsequently added to the column. Dioxin was eluted with 3 ml of 20% (V/V) benzene in hexane and the eluate concentrated to 1 ml.

A second chromatography used alumina that had been washed by saturating it with methylene chloride and activated at 225° C for 24 hours. A

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column, prepared as was the silica column, was stored at room temperature in a desiccater until used. After the column was wetted with hexane, the sample and a 1 ml hexane rinse of its container were transferred to the column. The alumina was eluted with two 3 ml portions of pesticide grade carbon tetrachloride, then with 4 ml of methylene chloride. These solvents were all used to rinse the container of the first-step eluate before being transferred to the alumina column. The separated methylene chloride fraction was concentrated under nitrogen as the volatile solvent was replaced with hexane. All other fractions were discarded.

The final chromatography used florisil that had been saturated with methylene chloride, activated at 165° C for 24 hours, and cooled in a vacuum desiccator. A 5 cm column in a disposable pipet plugged with glass wool was packed with 10 ml of hexane under light nitrogen pressure to remove air pockets. The sample, dissolved in 1 ml of hexane, was added to the column and its container rinsed with 1 ml of 8% (by volume) of methylene chloride in hexane. The column was eluted with 9 ml of the latter solvent mixture to remove 80-85% of the contaminating PCBs. A final elution with 8 ml of methylene chloride contained the TCDD and was collected in a centrifuge tube from which the solvent was evaporated to a small volume under a stream of dry nitrogen. The sides of the centrifuge tube were rinsed down with 1 ml of hexane and the volume was again reduced. After a final rinsing with 1 ml of hexane, the solvent was evaporated to a volume less than 100 microliters, the tube was closed with a teflon-lined screw cape and stored at -20° C.

Gas Chromatography/High Resolution Mass Spectrometry (GC/HRMS) Analysis. At the time of analysis, the sides of the centrifuge tube were washed thoroughly with approximately 100 microliters of hexane or isooctane using a graduated syringe. The solvent was allowed to evaporate during the washing until about 50 microliters remained and this volume was accurately measured. Three-fourths of the solution was replaced in the tube and the fourth remaining in the syringe was used for the initial analysis.

Gas chromatography employed a Perkin-Elmer Sigma II gas liquid chromatograph with a 6 foot by 1/4 inch O.D. glass column containing a Dow mixed phase packing. Typically it was operated at a helium flow rate of 15 cc per minute with the injector at 270°C. The column temperature was programmed for 1.5 minutes at 250° and then ramped at 10° C per minute to 300° C where it remained until the dioxin had eluted. The GC/HRMS interface was a direct coupling via a simple glass-lined stainless steel capillary held at an average temperature of 250° C. The typical retention time was 3.4 minutes with a peak width at 10% height of approximately 40 seconds.

The Kratos MS-5076 ultra high resolution mass spectrometer employed has an ultimate resolution equal to 180,000; it was tuned to a resolving power of 10,000 giving a 10% valley definition. The electron impact source was set at 70 eV ionizing energy and an accelerating voltage of 8 KV. The source was at 260° C. Data were acquired using the standard ion switching feature of the MS-50, i.e. dual ion monitoring.

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The first analysis was made monitoring one channel, m/z 321.8936, for the most abundant molecular ion of TCDD having natural isotopic elemental abundances. The second channel, m/z 327.8848, detected $^{37}Cl_4$-TCDD, the internal standard. The complete peak profiles were acquired at a band width of 3000 Hz by scanning at a frequency of about 2 HZ, corresponding in each case to a mass range of 300 parts per million (0.096 amu). The output of the spectrometer was accumulated over about 75 sweeps per channel using a Nicolet Model 1170 signal analyzer. The resulting signals were submitted to a three-point smoothing routine prior to being printed on an X-Y recorder.

Results were calculated by the internal standard "ratio method" in which standard samples containing known amounts of natural TCDD and the ^{37}Cl internal standard were analyzed throughout the testing period. A calibration curve was then prepared by plotting the ratio of the known weights of TCDD and internal standard against the ratio of signal intensities, i.e. intensity at m/z 321.8936 vs. intensity at m/z 327.8848. The content of TCDD in a test fat sample was calculated from the ratio of signal intensities at the two m/z values, reading the TCDD concentration from the calibration plot. The detection limit in the fat samples was obtained by multiplying the noise level by 2.5, considered to be the maximal amount of TCDD that could be present. The per cent recovery was measured using the absolute signal intensity for the internal standard and mass spectrometer response factors measured by analyzing standard solutions of the internal standard.

The second or validation analysis was performed by injecting a second aliquot from the graduated syringe into the GC/HRMS for any fat sample that showed detectable concentration of TCDD on the first analysis. For validation, the high mass channel was centered at 321.8936 and the low mass channel at 319.8965 for the second most abundant molecular ion of TCDD. All other conditions were as for the first analysis. The theoretic ratio of intensities, m/z 319.8965: m/z 321.8936, is 0.77.

The TCDD concentration in the validation sample was calculated based on the absolute signal intensity observed at m/z 321.8936, using response factors determined for the mass spectrometer by analysis of standard TCDD solutions. Based on the measured per cent recovery, the quantitation was adjusted to 100% recovery. The validation was considered acceptable if the observed ratio of signals was 9.77 ± 0.10.

RESULTS: All 34 fat samples have been analyzed and validated; the results are presented in Table I. Table II summarizes the data from Table I and discriminates between individuals who reported symptoms they felt were related to Orange exposure and those who reported no related symptoms.

The amount of TCDD ranged from none to 89 parts per trillion in the group of 23 persons reporting exposure to Orange. Six of 20 veterans (Codes 10, 12, 15, 25, 27 and 28) who reported exposure to defoliants in Viet

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Nam had concentration of TCDD that were greater than 2 parts per trillion above the detection limit. One such veteran had a sample that yielded equivocal results (Code 29) but has been included in Table II among those with a definite elevation. When the difference between concentration and detection limits is less than 3 parts per trillion (see Table I) assays results are questionable.

Among the 11 veterans, "controls", reporting no exposure to defoliants only one (Code 3) had a concentration of TCDD high enough to be accepted as certain. Three Air Force officers are shown separately in Table I. Although all had handled defoliants and TCDD within months of the biopsy and had been heavily exposed to Orange for years before that, none complained of symptoms despite levels above the certainty limits.

The 20 veterans reporting exposure all were selected as believing they had symptoms related to dioxin. None of the "controls" attributed ill effects to TCDD since none had known exposure to it. The assayed groups do not include an equally large group who were exposed to TCDD but have remained asymptomatic; the Air Force officers have these characteristics, however.

DISCUSSION AND CONCLUSIONS: The results demonstrate that GC/HRMS analysis can detect and measure very small amounts of tetra-chlorodibenzo-p-dioxin (TCDD) in human adipose tissue even long after the last known exposure. This makes available a research assay method that is rather unattractive because it requires surgical biopsy to obtain a sufficiently large fat sample and still suffers from technical difficulties.

Even though TCDD persists in the fat in amounts of a few parts per trillion it is unlikely to present a "ticking time bomb" as has been postulated. TCDD is found in fat in a higher concentration than in other body components and fat ordinarily accounts for only 1/7 to 1/5 of the total body weight of adult men. Even if all the body rather than adipose tissue alone contained as much as 100 parts per trillion of TCDD and released it simultaneously, the total amount would be much less than one one-hundredth of that required (on a per kilogram basis) to cause toxic symptoms in the most sensitive species.

These conclusions rest upon the accuracy of the assay employed for TCDD. The dependability of the GC/HRMS analysis has been checked by re-analyzing each extract and accepting only those results that are so validated.

There remain two sources of contamination to be considered, one in the assay laboratory, the other in the surgical procedure. The former can be dismissed if analyses at another laboratory confirm the results. To this end, portions of eight samples were sent to scientists at the Enviornmental Protection Agency (EPA). They employ different but complementary GC/MS procedures, using high resolution capillary column gas chromatography with lower resolving power mass spectrometry than in the present assays. Their results are in substantial agreement with those reported here.

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Although precautions were taken against contamination with interfering substances such as hexachlorophene during biopsy and tissue recovery, it is possible that TCDD may have been introduced in some unrecognized manner. This seems unlikely and unknown contact with the chemical during daily civilian activities is more probable.

Despite these reservations, the data suggest that service in Viet Nam increases the likelihood that a veteran's adipose tissue will contain a small but detectable amount of TCDD. The amounts are so tiny and the assay so delicate, however, that failure to detect TCDD does not indicate that there was no exposure to the compound a decade or more ago.

Symptoms, signs, and laboratory results on each of the volunteers are being collectd to attempt a correlation with the assay results.

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TABLE I

Analytic Results of TCDD Content of Human Adipose Tissue
(Expressed as Parts per Trillion)

Code	Wt. of Sample (Grams)	Concentration (Conc.)	Detection Limit (DL)	Difference (Conc. Minus DL) in PPT	% Recovery
A. Veterans Reporting Exposure to Orange in Viet Nam					
1	4.0	nd	5	0	50
6	9.5	5	3	2	65
8	10.4	5	3	2	50
9	10.5	nd	4	0	40
10	11.1	23	4	19	65
11	8.4	3	2	1	55
12	6.0	9	3	6	60
13	9.6	nd	2	0	80,60
14	3.9	4	3	1	65
15	7.1	7	4	3	50
16	5.2	nd	4	0	60
19	11.2	nd	3	0	20
24	4.4	nd	5	0	95
25	7.7	8	2	6	100
26	5.9	5	4	1	80
27	9.7	10	3	7	100
28	4.5	99	10	89	100
29	5.2	13*	5	-	60
30	6.8	nd	3	0	95
34	8.8	5	3	2	100
B. Air Force Officers with Intense Exposure to Orange					
2	11.6	5	2	3	50
3	7.7	4	1	3	85
4	10.2	6	2	4	45
C. Controls, Veterans Reporting No Exposure to Orange					
5	3.8	4,7	4	0	65
7	7.6	3	2	1	60
17	10.4	4	3	1	75
18	9.1	nd	4	0	30
20	9.9	5	4	1	50
21	10.0	6	3	3	35
22	1.4	nd	1	0	75
23	3.0	nd	6	0	100
31	8.8	nd	4	0	50
32	9.3	4	4	0	60
33	2.2	nd	7	0	100

*Results of validation indicate that material detected may not be TCDD

TABLE II

Summary of Results of TCDD Analysis
of Human Adipose Tissue

(Denominator is total number of individuals in a group; numerator is number of individuals with more than 2 parts per trillion of TCDD in the fat, e.g., 7/20 indicates that seven of the 20 veterans with symptoms and exposure had 3 parts per trillion or more)

	Viet Nam Service	No Viet Nam Service
Symptoms	7/20	0/1
No Symptoms	3/3*	1/10
Total	10/23	1/11

* 3 Air Force officers with heavy and recent exposure