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Analysis for 2,3,7,8-Tetrachlorodibenzo-p-dioxin in Adipose Tissue from Vietnam Veterans.

by

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ABSTRACT

Tetrachlorodibenzo-p-dioxin (TCDD) has been detected at levels ranging from 20-173 parts-per-trillion in adipose tissue from three Vietnam veterans who were "heavily exposed" to Herbicide Orange. The detection corresponded to a single isomer, having the characteristics of 2,3,7,8-TCDD, and was validated in interlaboratory studies for two of the three tissue samples. Tissue samples from other Vietnam veterans and from controls also contained 2,3,7,8-TCDD at levels below 20 parts-per-trillion. These findings are in accord with uptake and long term storage of 2,3,7,8-TCDD by veterans who experienced a heavy exposure to Herbicide Orange. Furthermore, they may signal accumulation of 2,3,7,8-TCDD in some members of the U.S. population.

Aerial defoliation in Vietnam carried out by the United States military from 1962-1970 made use of Herbicide Orange, a 1:1 mixture of the herbicides 2,4,5-T and 2,4-D. The 2,4,5-T contained a contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) at levels sufficient to produce a mean concentration of about 2 parts-per-million in the defoliant.[1] The 2,3,7,8-TCDD, in addition to being extremely toxic, is known to be carcinogenic and teratogenic in animal tests.[2]

Vietnam veterans now claim adverse chronic and delayed health effects related to their exposure to Herbicide Orange. These claims and recent publicity regarding the military defoliation program have increased public awareness of the potential health effects of exposure to 2,3,7,8-TCDD, especially among Vietnam veterans.

We report here the results of a preliminary study designed to determine the range of TCDD levels that might exist in the tissue of veterans. Specifically, we wished to determine if 2,3,7,8-TCDD could be detected at levels above 1-5 parts-per-trillion. Adipose tissue was chosen as the preferred sampling medium because of its good accessibility (compared to other tissue) and because 2,3,7,8-TCDD is known to accumulate in this tissue in other species.[3,4] Secondly, we wished to examine whether any correlation exists between exposure (assigned by the Veteran's Administration[5]) and observed levels. The existence of a correlation could signal the need for additional monitoring studies.

In order to have confidence in the results generated from test samples, it is necessary to demonstrate the capability to conduct analyses of TCDD at low ppt levels. This has been demonstrated in the laboratories involved in this analytical work by means of blind validation studies in which coded spiked samples of beef fat and human milk were analyzed.[6,7] Additional validation of the methodology used in this study for low levels of TCDD has been demonstrated at the 1-100 part-per-trillion range using human and beef adipose tissue (see Table 1).

The Veterans' Administration supplied thirty coded samples of adipose tissue which had been surgically removed from the abdominal area of "exposed" veterans and control subjects who had not served in Vietnam. Additional tissue was taken from three U.S. Air Force officers with several years experience in TCDD research. Particular care was exercised, even in tissue removal, to avoid any potential contamination. The samples were placed in rigorously cleaned glass

vessels, frozen, shipped in dry ice, and stored in a frozen state (-20°C). No disinfectants containing hexachlorophene or other trichlorophenol-based materials were used.

Approximately 5 to 10 grams of tissue were used, when available, for each analysis. A known amount, generally 2 ng, of internal standard (either ^{37}Cl-2,3,7,8-TCDD or ^{13}C-2,3,7,8-TCDD) was added to the adipose tissue. The sample was digested in alcoholic potassium hydroxide followed by extraction with hexane to remove TCDD. The hexane extract was washed with concentrated sulfuric acid, neutralized, dried, and concentrated. The final stage of clean-up involved the use of three short-column liquid chromatography steps (silica gel, alumina, and Florisil).[9] Gas chromatography/high resolution mass spectrometry (as described previously[8,10]) was employed for quantitation of 2,3,7,8-TCDD and coeluting isomers. Signal profiles were obtained at a mass resolution of 10,000 for m/z 321.8936, the most abundant molecular ion for TCDD, and for the internal standard mass by signal averaging for approximately 100 sec. commencing with the appearance of the co-eluting internal standard 2,3,7,8-TCDD.

Extracts which contained materials giving signals greater than 2.5 times noise at the exact mass of TCDD (i.e. 321.8936 ± 0.0020) over the integration period discussed above were reanalyzed. For the second analysis, signal profiles of m/z 321.8936 and m/z 319.8965 were monitored over the elution period of 2,3,7,8-TCDD (determined by injection of standard solutions). A positive detection was reported if signals were observed above the detection limit (2.5 times noise) and if their intensity ratio was 1.0:0.78 ± 0.10 , which is consistent

with the presence of four chlorine atoms in the molecule. Samples meeting all criteria except the correct isotope intensity ratio have been considered to contain "not detectable" levels of TCDD. For these samples, we judged that the presence of TCDD is not disproved by the observation of an incorrect isotope ratio at these low concentrations; rather, the presence of TCDD is not confirmed.

For the first stage of analyses, both extraction and quantitation of all samples were conducted at the University of Nebraska, Midwest Center for Mass Spectrometry (see Table 2 for results).[11] Two of the three samples of tissue from veterans considered "heavily exposed" to Herbicide Orange[12] were found to contain the highest levels of TCDD detected in this study. The recovery (20%) of internal standard for the tissue sample of the third subject was unacceptably low. We consider the levels to be minimum values, and the higher level, in cases of replicate analysis, is judged to be more accurate. This is because the internal standard signal for ^{37}Cl-2,3,7,8-TCDD at m/z 327.8849 is susceptible to an interference from m/z 327.8775 ($C_{12}H_5{}^{35}Cl_3{}^{37}Cl_2$), a molecular ion of a polychlorinated biphenyl, which could enhance the intensity of the standard and lead to the calculation of too low a value for the TCDD using the internal standard method of quantitation.

Three of five samples of tissue from veterans having "light exposure" were found to contain TCDD, as were tissue from two of the three Air Force officers who have done extensive research with environmental and biological samples containing 2,3,7,8-TCDD. Seven of thirteen tissue samples from other Vietnam veterans were found to contain 2,3,7,8-TCDD or coeluting isomers at levels between 3 and 13

parts-per-trillion.

Samples taken from veterans who had no service in Vietnam (controls) also showed low levels of TCDD (four of ten were judged to be "positive" at levels between 4 and 8 parts-per-trillion).

In view of the limited isomer specificity [14] of the methodology used at the University of Nebraska (UN-L) [13] and the need to increase certainty by interlaboratory validation of reported detections at these low levels, a subset of the tissue samples was reextracted and/or reanalyzed at other laboratories (see Table 3). The interlaboratory validation was done in two ways. First, extracts from the first stage of analyses at UN-L were reanalyzed at the Environmental Protection Agency laboratory at Research Triangle Park (RTP) using capillary column gas chromatography/high resolution mass spectrometry (reported as UN-L/RTP in Table 3). Second, portions of the tissues stored in the University of Nebraska laboratory were transmitted to the EPA Toxicant Analysis Center (TAC) for extraction and clean-up. The extracts were then forwarded to the Research Triangle Park laboratory for analysis (reported as TAC/RTP in Table 3). All samples were coded at the University of Nebraska and their identities were not known to the workers at TAC or RTP.

The tissues were extracted at TAC using methods similar to those employed at the University of Nebraska.[6] The quantitation conducted at the RTP laboratory made use of capillary column GC and peak-top monitoring (rather than peak profiles) at a mass resolution of 6000-10,000,[6] a method more specific for TCDD isomers than that employed at the University of Nebraska. All detections reported in Table 3 were for a material having an identical GC retention time as 2,3,7,8-TCDD

and no other isomers were detected at levels greater than 10% of the level corresponding to 2,3,7,8-TCDD. Some small degree of uncertainty remains that the material detected is the 2,3,7,8-TCDD isomer because standards for all 22 TCDD isomers were not available at the RTP laboratory.

Based on the interlaboratory validation studies,it is now confirmed that two of three samples from veterans designated as having "heavy exposure" were found to contain measurable amounts of 2,3,7,8-TCDD at levels higher than any other participant's tissue in this study. Adipose tissue from the third "heavily exposed" veteran is a "possible positive"; i.e., the positive detection made by RTP scientists was not validated by the group at Nebraska, presumably because of the problem with recovery.

Both the USAF officers' adipose tissues, which were examined in the interlaboratory validation, definitely contained 2,3,7,8-TCDD, but at lower levels than the tissue of "heavily exposed" veterans.

Definite detections of 2,3,7,8-TCDD were made in the analyses of adipose tissue from other exposed veterans and veterans who never served in Vietnam (controls). The detection of 46 ppt for VA-21 is considered an aberrantly high value based on a comparison with four other analyses reported in Table 3. Ignoring this value, the highest level detected in these two groups (20 ppt) coincides with the lowest detection made for the "heavily exposed" veterans. These observations may signal base-line accumulation of TCDD in some members of the general U.S. population.

There is good agreement of the results obtained in both mass spectrometry laboratories particularly in terms of the assignments of

whether the sample contains TCDD. Differences in concentrations were found for repeated analysis of samples containing the higher levels of TCDD. These differences appear to be systematic; for example, the concentrations determined by the Nebraska workers may be low as explained above. This lack of agreement is expected considering the difficulty of performing extractions and analyses at these low concentrations. Accordingly, we do not consider the differences obtained for a given sample to be significant.

Because TCDD could be detected in some control samples, a simple statistical analysis[16] was used to test the significance of the results. A contingency table for the hypothesis of independence was used to test the supposition that "concentration levels of TCDD in adipose tissue of test individuals is unrelated to exposure to Herbicide Orange". The exposure classifications as previously listed were used except the Air Force scientists were excluded. The concentrations of TCDD were classified in four categories: (1) "less than four parts-per-trillion or not detected", (2) "4 to 10 parts-per-trillion", (3) "11 to 20 parts-per-trillion", and (4) "greater than 20 parts-per-trillion". The concentrations of TCDD used in the statistical test were averages of all determinations made in both laboratories with the following exceptions. We did not use reports of "not detected" having detection limits greater than or equal to positive detection made in separate analyses (see VA 8, 9, 19, 34). The "not detected" assignments of samples having low recoveries of internal standard (VA 18 and 19, Table 2) were not used. Finally, determinations which appeared to be positive but yielded incorrect isotope ratios (see Table 2) were considered as "not detected" except

when another analysis showed that the sample contained TCDD. The third concentration classification was chosen to encompass the "heavily exposed" veteran's tissue which was considered "possible positive" (VA 19) and the control sample having the highest concentration of TCDD (VA 20). The levels of 2,3,7,8-TCDD in tissues of the other two "heavily exposed" veterans were found to be considerably higher in all analyses, and they were the only results which fall in the fourth category. The chi-square test showed that the hypothesis of independence was false at the 95% confidence level, indicating a correlation between degree of exposure to Herbicide Orange and tissue levels of 2,3,7,8-TCDD.

In conclusion, Vietnam veterans designated by the Veteran's Administration as "heavily exposed" to Herbicide Orange carry low levels of 2,3,7,8-TCDD in adipose tissue,as validated in interlaboratory analytical studies. The 2,3,7,8-TCDD levels found in these veterans are higher than for other exposed veterans or for the controls. The observations that the TCDD observed is a single isomer, and likely the 2,3,7,8-isomer found in 2,4,5-T, and that the levels in the "heavily exposed" group are higher than those observed in any of the other study cases are in accord with an interpretation that the exposure occured in Vietnam. We emphasize that the confidence ascribed to this conclusion can be increased (or decreased) by conducting a more thorough investigation which would include a larger sample of Vietnam veterans "heavily exposed" to Herbicide Orange.

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11. A number of method blanks (14) were extracted and analyzed with actual tissue samples so that one blank for approximately every three samples (including repeats) was examined. No TCDD was detected in the blanks at at an average detection limit of 4 and a range of 1 to 11 parts-per-trillion.

12. The classification as to degree of exposure was made by the U.S. Veterans Administration (see reference 5).

13. The packed column used in these studies has been evaluated by Dow Chemical Company scientists in terms of TCDD isomer specificity (see reference 15). In our hands 2,3,7,8-TCDD, for example, could be separated from 1,3,6,8-TCDD with base line resolution.

14. Another interlaboratory study, TAC/RTP, was conducted prior to the two reported in table 3. Analysis of method blanks showed that the glassware in the TAC laboratory was contaminated with 2,3,7,8-TCDD and other isomers at the time of the study. None of these results are reported here. All glassware was replaced for the

later studies, which yielded the results given in table 3. Actual adipose samples VA-9 and 10 were presumably contaminated during the time of the first extraction at TAC as they were found to contain 160 and 230 ppt respectively in the second TAC/RTP validation run (coded TCDD TAC/RTP[c]). These results are also not reported in Table 3 because they are clearly not consistent with results of other analyses of these samples. Samples VA-26 and VA-31 were not present in the TAC laboratory at the time of contamination. They and additional portions of tissue samples for the third validation run (Coded TAC/RTP[d]) were transmitted to TAC after the laboratory contamination had been removed.

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Table 1. Analysis of Control Samples for TCDD.

Nature of Sample	Sample Wt. (g)	Spike (ng)	Concentration Added (ppt)[1]	Concentration Found (ppt)	Detection Limit	Percent Recovery[2]
Human Fat	14.80	2.00	0	nd	3	40
Human Fat	14.80	2.05	6	9	3	40
Human Fat	14.70	2.00	16	20	4	45
Human Fat	15.55	2.00	38	41	4	40
Beef Fat	9.91	1.00	2	$1 \pm .1$ [3]	0.5	120
Beef Fat	10.44	1.00	96	75 ± 5 [4]	3	60

1. ppt = parts-per-trillion

2. recovery of internal standard

3. average of two analyses

4. average of three analyses

Table 2. Results of Stage 1 Analysis of TCDD in Human Adipose Tissue[1]

VA Code Number	Concentration (ppt)[2]	Detection Limit (ppt)	Percent Recovery	Ratio[3]
"Heavily Exposed Veterans"				
10	23	4	65	.85
10	35	9	100+	.75
19	ND[2]	3	20	---
26	99	10	90	.77
26	63	6	45	---
"Lightly Exposed Veterans"				
1	ND	5	50	---
13	ND	2	80	---
15	7	4	50	.88
28	7	5	95	---
28	8	6	40	.78
34	5	3	100	.85
"Possibly Exposed Veterans"				
6	5	3	65	.90
8	5	3	50	.90
9	ND[1]	3	40	---
11	3	2	55	.77
12	9	3	60	.88
14	4	3	65	.74
16	ND	4	60	---
24	5	3	80	.71
24	5	4	45	---
25	12	4	45	---
25	10	3	100+	.78
27	ND	6	100	---
29	13	5	60	.88
30	ND	3	95	---
"Controls"				
5	4	4	65	1.02
7	3	2	60	.92
17	4,3[4]	3	75	.84
18	ND	4	30	---
20	5	4	50	.86
21	6	3	35	1.07
23	8	2	100	.78
23	6	3	55	---
31	7	4	50	.98
32	4	4	60	.74
33	14	7	100	.94

Table 2 continued.

VA Code Number	Concentration (ppt)[2]	Detection Limit (ppt)	Percent Recovery	Ratio[3]
"USAF Scientists"				
2	5	2	50	.77
3	4	1	85	.94
4	6	2	50	.76

1. Sample sizes ranged from 2.2 - 11.6g for each extraction. Internal standard amounts used varied from 2.0 - 2.6ng/extraction.

2. ppt = parts-per-trillion; ND = not detected.

3. Ratio of intensities of m/z 320 and m/z 322. Acceptable values are 0.78 $\pm$ 0.10.

4. Duplicate analysis of same extract.

Table 3. Results of Interlaboratory Validation Studies.

VA Code	UN-L/UN-L[a]	UNL/RTP[b]	TAC/RTP[c]	TAC/RTP[d]	UN-L/UN-L[e]
"Heavily Exposed Veterans"					
VA-26	63,99	----	173	----	----
VA-10	23,35	36	h	86	----
VA-19	ND(3)[e]	----	----	20	ND(29)
USAF Researchers					
VA-3	4[g]	3	10	----	----
VA-2	5	----	----	24	----
Other Vietnam Veterans					
VA-13	ND(2)	ND(0.2)	ND(7)	----	----
VA-8	5	3	5	----	----
VA-9	ND(3)	3	h	ND(7)	----
VA-15	7	----	----	18	----
VA-34	5	----	----	ND(5)[f]	----
Controls					
VA-17	4,3	----	20	14	----
VA-18	ND(4)[f]	5	8	----	----
VA-21	6[g]	3	12	46	9
VA-31	ND(4)	----	ND(3)	----	----
VA-20	5	----	----	19	20

Notes:

(a) Extracted at UN-L/Analyzed at UN-L (from Table 2). The values given in parentheses are the detection limits.

(b) Portion of the Extract from UN-L/Analyzed at RTP

(c) Extracted at TAC/Analyzed at RTP

(d) Another portion of tissue shipped from UN-L, extracted at TAC/Analyzed at RTP

(e) Extracted at UN-L/ Analyzed at UN-L. Results obtained with knowledge of the code.

(f) Poor recovery of internal standard (<40%).

(g) Isotope ratio for m/z 320 and m/z 322 not correct.

(h) See footnote 14 regarding these samples.