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February 12, 1980

Dr. Lawrence Hobson, M.D., Ph.D.
Veterans Administration Central Office
810 Vermont N.W.
Washington, DC 20420

Dear Larry:

Enclosed please find my final report on the analytical work on human adipose. I have included some of the EPA results in Appendix I. These results only validate our analysis. Their extractions are not yet complete, and we should wait for them because they should serve as a validation of the extraction/clean-up. Those data will be submitted to you as Appendix II.

I have sent a copy to Lyndon Lee and propose to send one to Mike Delarrco at EPA when their work is done.

As a short follow-up to our work, we plan to look at the feasibility of analyzing 0.5 g sample sizes. If successful, that would permit the use of this methodology for wider screening.

It has been a pleasure working on this. I will await your reply and the permission to publish the results in Science or the like.

Best regards,

Michael L. Gross
Professor and Director

MLG: lc
c.c: Dr. Lyndon Lee
Enclosure

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Report to

Veterans Administration

c/o Lawrence B. Hobson, M.D., Ph.D.

Trace Analysis of Tetrachlorodibenzo-p-dioxin (TCDD) in Human Adipose

by

Michael L. Gross
Professor and Director

2-13-80
Date

c.c: Dr. Lyndon Lee

Introduction

This is a report of the analytical data obtained for the analysis of 2,3,7,8-tetrachlorodibenzo-p-dioxin in human adipose tissue of Vietnam war veterans. The contents of the report are as follows:

1. Sample Extraction Procedure for Tissue: a description of the clean-up, work-up procedure.

2. Gas Chromatography/High Resolution Mass Spectrometry Analysis: a description of the actual analysis of the tissue extract.

3. Results: all the relevant data for each analysis reported in tabular form.

4. Discussion: a discussion of the actual data.

5. Conclusion: a consideration of the number of "positive" detections found.

Because of the difficulty of analyzing chemical substances at the parts-per-trillion level, we requested collaboration from the Environmental Protection Agency. With their support, a method validation approach has been developed and employed successfully in the analysis of human milk for TCDD. For application to this study, samples selected after consultation with Dr. James Norman, the statistician assigned to the project, were sent to Dr. Aubry Dupuy, Environmental Protection Agency, Pesticide Monitoring Laboratory, NASA/NSPL, Bldg. 1105, Bay St. Louis, Mississippi 39520, and worked up employing a method nearly identical to our own. The extracts were then submitted to Mr. Robert Harless at the Research Triangle Park Laboratory for GC/MS analysis. In addition, portions of some of our extracts were submitted

to Harless. The results of both of these collaborative efforts are given in Appendix I and Appendix II (the data for Appendix II were not available at this writing and will be forwarded to the VA at a later time).

Sample Extraction Procedure for Tissue

A 1-10g sample was accurately weighed and spiked with a known amount (2.0 - 2.5 ng) of Cl^{37}-TCDD. It was then saponified in 15 ml of ethanol* and 30 ml of 40% acqueous KOH in a reflux apparatus for 60 minutes with stirring. The sample should be completely hydrolyzed before terminating the saponification.

The solution was transferred to a 250 ml separatory funnel and diluted with 20 ml of ethanol and 40 ml of water and extracted four times with nanograde hexane. The first extraction was done with 25 ml of hexane, shaking vigorously for one minute. The lower aqueous layer was removed to a clean beaker, and the upper hexane layer was decanted to a 125 ml separatory funnel. The aqueous layer was then extracted three times more with 15 ml portions of hexane, each time adding the hexane to the 125 ml separatory funnel. The combined hexane extracts were washed with 10 ml water to remove excess base.

The combined hexane extracts were washed 4 times with 10 ml concentrated H_2SO_4, or until both layers were clear. As many as 8 extractions may be necessary, depending upon the sample. Again the hexane was washed with 10 ml water. The hexane layer was decanted to a 2 ounce jar and concentrated under a stream of dry nitrogen to approximately one ml.

Three chromatography steps were done, the first being a silica gel column. No activation of silica was necessary. A 5 cm column was prepared using a disposable pipet plugged with glass wool. The silica was capped with 1/4 cm anhydrous sodium sulfate to remove water, and then wetted with hexane. The sample, dissolved in 1 ml of hexane, was transferred to the column. A second ml of hexane was used to rinse the jar and was subsequently added to the column. Dioxin was eluted with 3 ml of 20%

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*All solvents are of the highest grade and suitable for residue analysis.

(V/V) benzene in hexane. All the eluate was collected in another 2 ounce jar and concentrated to a volume of 1 ml.

Alumina was washed by saturating with methylene chloride, removing excess solvent, then activating at 225° C for 24 hours. A column was prepared in the same manner as the silica column above. The column was cooled to room temperature in a dessicator before use.

Hexane was used to wet the column before transferring the sample. The jar was again rinsed with one ml of hexane which was transferred to the column. The alumina was eluted with two 3 ml portions of pesticide grade CCl_4, then with 4 ml of CH_2Cl_2. These solvents were used to rinse the jar before being transferred to the column. The methylene chloride fraction was collected in a clean 2 ounce jar and concentrated under nitrogen while replacing the volatile CH_2Cl_2 with hexane. All other fractions can be discarded.

The final step was florisil chromatography. The florisil was saturated with methylene chloride and activated in an oven at 165° C for 24 hours. The packing was allowed to cool in a vacuum dessicator. A five cm column was prepared in a disposable pipet plugged with glass wool. The column was packed with 10 ml of hexane under light nitrogen pressure, in an attempt to remove all air pockets.

The sample, dissolved in one ml of hexane, was added to the florisil column. The container was rinsed with one ml of 8% (by volume) methylene chloride in hexane. The column was eluted with nine ml of 8% CH_2Cl_2 in hexane (which removed 80-85% of the PCB's) and then with eight ml of CH_2Cl_2. The dichloromethane fraction, which contained the TCDD, was collected in a centrifuge tube, and the solvent was evaporated to a small volume under a stream of dry nitrogen. The sides of the centrifuge tube were rinsed down with one ml of hexane and again the volume was reduced. The tube was rinsed a final time with one ml of hexane and the solvent evaporated until the volume was less than 100µℓ. The centrifuge tube was capped with a teflon-lined screw cap and and stored in a freezer at about -20°C until analysis.

List of Materials Used in Tissue Extractions

Acetone, OmniSolv*, MCB

Benzene, OmniSolv, MCB

Carbon tetrachloride, OmniSolv, MCB

Ethyl alcohol, OmniSolv, MCB

Hexane, OmniSolv, MCB, non UV

Methylene chloride, OmniSolv, MCB

Sulfuric acid, concentrated, analytical reagent, Mallinckrodt

Water, distilled in glass

Potassium hydroxide, analytical grade, Mallinckrodt

Sodium sulfate (anhydrous), analytical grade, Fisher

Aluminum oxide, neutral, activity grade I, Woelm Pharma

Florisil, 60-100 mesh, Fisher

Silica gel, 60-200 mesh, reagent grade, Baker Chemical Co.

Dry nitrogen (boil-off from liquid N_2)

*All OmniSolv line solvents are distilled in glass, suitable for chromatography and residue analysis.

Gas Chromatography/High Resolution Mass Spectrometry (GC/HRMS) Analysis

Sample Handling

At the time of analysis, the side of the centrifuge tubes was washed thoroughly with approximately 100µl of hexane or isooctane using a graduated syringe. During the washing, the solvent was allowed to evaporate until a volume of ∿50µl remained. This remaining volume was accurately measured; usually three-fourths was replaced in the centrifuge tube, and the fourth remaining in the syringe was used for the gas chromatography/mass spectrometry analysis.

Mass Spectrometer

A Kratos MS-5076 ultra high resolution mass spectrometer was used for this analysis (ultimate resolution = 180,000). The mass spectrometer was interfaced via a direct coupling to a Perkin Elmer Sigma II gas liquid chromatograph. Data acquisition was accomplished with a Nicolet Model 1170 signal averaging computer.

Gas Chromatography, [Samples 1-22 and 23-34(first-time analyses)]

The column was a 6' x 1/4" O. D. glass containing a Dow mixed phase packing. Typical operating conditions were: Helium flow rate of 15 cc/min; injector 270° C: column temperature program 1.5 min at 250° and then ramped at 10° C /min to 300° C and held there until the dioxin had eluted. The GC/MS interface was a simple glass lined stainless steel capillary and was held at an average temperature of 250° C. Typical retention time was 3.4 minutes (peak width at 10% height approximately 40 seconds).

(23-34 final analyses) The column was a 6' x 1/4" O. D. glass containing an OV-3 packing. Typical operating conditions were: Helium flow rate of 8 cc/min; injector 275° C: column temperature program 1.0 min at 275° and then ramped at 10° C/min to 300° C and held there until the dioxin had eluted. The GC/MS interface was a simple

glass lined stainless steel capillary and was held at an average temperature of 250° C. Typical retention time was 2.6 minutes (peak width at 10% height approximately 40 seconds).

Mass Spectrometer Conditions

The electron impact source was used at 70eV ionizing energy and an accelerating voltage of 8KV. The source was set at 250° C. The instrument was tuned to a resolving power of 10,000 (10% valley definition).

Data were acquired using the standard ion switching feature provided with the MS-50 (dual ion monitoring). The first analysis was made monitoring one channel m/z 321.8936 (the most abundant molecular ion of TCDD having natural isotopic elemental abundances) and m/z 327.8848 ($^{37}Cl_4$-TCDD, the internal standard) on the second channel. The complete peak profiles were acquired at a bandwidth of 1000Hz by scanning of a frequency of about 2Hz, corresponding in each case to a mass range of 300 ppm (0.096amu). The output of the mass spectrometer was accumulated over about 75 sweeps per channel using a Nicolet Model 1170 signal averager. The resulting signals were submitted to a three-point smoothing routine prior to print out on an X-Y recorder.

Calculation of Results

Quantitation was achieved by employing the internal standard "ratio method". Throughout the analysis period, standard samples containing TCDD and internal standard were analyzed. From these results, a calibration curve can be prepared by plotting ratio of the weights of TCDD and internal standard versus the ratio of signal intensities (intensity at m/z 321.8936; intensity at m/z 327.8848). Residues of TCDD in actual samples were obtained by measuring the ratio of the signal intensities at m/e 322 and at 328 (internal standard) and reading the concentration of TCDD from the calibration plot. The detection limit in the actual samples was obtained by multiplying the noise level by 2.5 which was considered the maximum amount of TCDD which could be present in the sample.

The percent recovery was measured using the absolute signal intensity for the internal standard and mass spectrometer response factors measured by analyzing standard solutions of internal standard.

Validation

Samples which showed detectable concentrations of TCDD or which were questionable were reanalyzed by removing a second aliquot and reinjecting onto the GC/HRMS (see data table). For this validation, the high mass channel is centered at 321.8936 and the low mass channel at 319.8965, the second most abundant molecular ion of TCDD. All other conditions were as reported above. The theoretical ratio of intensities is 0.78 (m/z 319.8965: m/z 321.8936).

The analysis permits us to calculate a concentration of TCDD based on the absolute signal intensity observed at m/z 321.8936 using response factors determined for the mass spectrometer from analysis of standard solutions of TCDD. Based on the percent recovery measured above, the quantitation may be adjusted to 100% recovery.

Validation of TCDD is considered acceptable if the observed ratio of signals is 0.78 ± 0.10.

RESULTS: Analysis of TCDD in Human Tissue by GLC/High Resolution Mass Spectrometry

Table 1

ID# (VA-Code)[1]	Sample Wt. (g)	ng Spike	Conc. TCDD (ppt)[2]	Detection Limit	Percent Recovery	Conc.[3]	$i_{320/322}$
1	2.3	1.8	nd	11	100	--	--
	4.0	2.0	nd	5	50	--	--
2	11.6	2.0	5	2	50	4	.77
3	7.7	2.5	4	1	85	2	.94[4]
4	10.2	2.0	6	2	50	4	.76
5	3.7	2.0	9	5	5	6	.92[4]
	3.8	2.0	4	4	65	5	1.02[4]
6	9.5	2.0	5	3	65	2	.90[4]
	9.5	---	-	-	--	nd(<2)	--[5]
7	7.6	2.0	3	2	60	2	.92[4]
	7.6	---	-	-	--	nd(<1)	--[5]
8	10.4	2.0	5	3	50	2	.90[4]
	10.4	---	-	-	--	nd(<1)	--[5]
9	6.4	2.0	nd	12	100+	--	--
	10.5	2.0	nd	3	40	--	--
	10.5	2.0	nd	4	40	1	.64[4]
10	9.3	2.3	12	2	100+	--	--
	9.3	2.3	16	4	100+	--	--
	10.7	2.0	35	9	100+	21	.75
	11.1	2.6	23	4	65	17	.85

ID# (VA-Code)	Sample Wt. (g)	ng Spike	Conc.[2] TCDD (ppt)	Detection Limit	Percent Recovery	Conc.[3]	$i_{320/322}$
11	8.4	2.0	3	2	55	2	.98
	8.4	---	-	-	--	1	.77
12	6.0	2.0	9	3	60	13	.88
13	9.6	2.0	nd	2	60	--	---
	9.6	2.0	nd	2	80	--	---
14	3.9	2.0	4	3	65	4	.74
15	7.1	2.0	7	4	50	8	.88
16	5.2	2.0	nd	4	60	--	---
	5.2	2.0	nd	8	25	--	---
17	10.4	2.5	3	3	65	2	.84
	10.4	2.5	4	3	75	3	.84
18	9.1	2.0	nd	4	30	--	---
19	6.4	2.0	nd	15	20	--	---
	11.2	2.0	nd	3	20	--	---
20	9.9	2.0	5	4	50	5	.86
21	10.9	2.0	6	3	35	2	1.07[4]
23	11.6	2.0	6	3	55	--	---
	7.7	2.0	8	2	100	8	.78
24	9.8	2.0	5	4	45	--	---
	5.9	2.1	5	3	80	3	.71

ID# (VA - Code)	Sample Wt. (g)	ng Spike	Conc. TCDD (ppt)[2]	Detection Limit	Percent Recovery	Conc.[3]	$i_{320/322}$
25	12.2	2.0	12	4	45	--	---
	9.7	2.0	10	3	100+	11	.78
26	8.7	2.0	63	6	45	--	---
	4.5	2.1	99	10	90	100	.77
27	4.6	1.9	nd	11	30	--	---
	3.0	2.2	nd	6	100	nd(<5)	---
28	6.2	2.0	8	6	40	3	.78
	4.4	2.0	7	5	95	--	---
29	8.4	2.1	nd	6	45[6]	--	---
	5.2	2.1	13	5	60	8	.88
30	6.6	2.1	9	4	45[7]	--	---
	6.8	2.2	nd	3	95	nd(<5)	---
31	9.4	2.0	4	2	65	--	---[4]
	8.8	2.0	7	4	50	3	.98[4]
32	12.2	2.0	nd	5	20	--	---
	9.5	2.0	4	4	60	5	.74
33	5.5	2.2	7	6	45	--	---[4]
	2.2	2.2	14	7	100	5	.94[4]
34	11.4	2.2	4	4	25	--	---
	8.8	2.0	5	3	100	5	.85

FOOTNOTES:

1. Sample numbers from October 1, 1979, document "Fat Biopsy Cases". Sample #22 was never received.
2. ppt = parts-per-trillion
3. via the absolute intensity of 322
4. Cannot be considered positive because of poor validation.
5. Contaminant removed on repeat of analysis.
6. poor recovery and poor cleanup
7. Probable lab contamination. Additonal sample worked up and found nd.

Discussion

The calculated concentrations given in Table 1 (first concentration value) are independent of the percent recovery and instrument sensitivity because of the use of the internal standard, $^{37}Cl_4$-TCDD, method. The calculated detection limits (2.5 x noise level) are somewhat high in these analyses primarily because of the small sample sizes and the chemical interferences in the sample extract. The percent recovery is based on the response of the mass spectrometer to the internal standard added before the work-up, and thus, this number will change with variations in instrument sensitivity and sample delivery into the gas chromatograph inlet.

We find that the reliability for a percent recovery measurement for a sample changes as the sample is handled for repeated analyses. This is probably due to small losses of sample on the glass container and to changes in concentration from solvent evaporation. The method of quantitation is not susceptible to these problems.

The concentration determined in the validation study is based solely on m/e 320 and m/e 322 and not on an internal standard. It will be low by a factor equal to the reciprocal of the percent recovery expressed as a fraction. This measurement is made primarily to obtain the isotope ratio from the signal intensity at masses m/e 320 and 322, as confirmation that the substance with the same exact mass as TCDD is in fact TCDD. Thus, the concentration determined by the 322/328 measurement is considered to be more reliable.

Some of the sample analyses gave positive detections but an incorrect isotope ratio was observed in the validation study (3,5-9,21,31,33). Samples 6-8 also gave a "not detected" on at least one occasion, when the chromatography was sufficient to remove the interfering substance, and this demonstrates that the observed signal was not due to TCDD.

Interfering ions can also lead to the designation of true positives as "not detected" when the chromatography is insufficient to remove contamination. For these analyses, the location of the signal base line is difficult and a TCDD signal may be buried in noise of an adjacent signal. This occurred for sample #29 and was readily observed because the entire mass range adjoining the TCDD showed interfering signals. The sample was reanalyzed with particular care to correctly perform all liquid chromatography steps, and the TCDD was "unmasked" in the repeat to give a clear signal with S/N greater than 2.5.

Another difficulty arises when glassware used in the cleanup is unintentionally contaminated with native TCDD. We believe this may be the case for sample #30. The repeat analysis of another newly worked-up sample of #30 indicated a "not detected" level of TCDD.

At the lower parts-per-trillion levels (1-10ppt), only one significant figure is used in reporting the results. However, the use of a criterion of 2.5:1 for the signal-to-noise (S/N) ratio to determine the detection limit implies the ability to distinguish the difference in signal and noise levels to two significant figures. The use of a 3:1 S/N may be more reasonable.

In the conclusion, we list the samples which are considered positive as a result of our research.

Conclusion

The following criteria must be met for a sample to be judged to contain TCDD.

1. The gas chromatography retention time must be correct.
2. The suspected material must give a signal at 321.8936 ± 0.0015. (This is determined by real-time peak matching of a suspected material and the TCDD-$^{37}Cl_4$.)
3. The signal intensity ratio for m/e 320/322 must be 0.78 ± 0.10.
4. The exact mass of m/e 320 must be 319.8965 ± 0.0015 using m/e 321.8936 as a standard.
5. The signal must be greater than 2.5 (3.0) times the noise level.

Using these, we judge the following samples to contain detectable concentrations of TCDD:

2,4,10,11,12,14,15,17,20,23,24,25,26,28,29,32,34

However, a 3/1 S/N criterion may be more appropriate. Adopting this, we assign the following as containing detectable levels of TCDD:

2,4,10,11,12, 15, 20, 23, 24, 25, 26, 28, 29, 34

An important question is the exact nature of the TCDD isomer; i.e. is the detected TCDD the 2,3,7,8-isomer? This question is impossible to answer unambiguously unless all 22 isomers are available as standards. Furthermore, the gas chromatography must be sufficiently specific to separate or distinguish the 22 isomers. Of course, this cannot be evaluated without the reference isomers which are not available.

The gas chromatography employed at the University of Nebraska is packed column which can be used to distinguish some of the isomers, but certainly not all. The gas chromatography at Research Triangle Park/EPA-laboratory is capillary column. Using their method, most of the known TCDD isomers can be separated.

For the experiments reported in the results section and in Appendix I, the retention time of the observed signal was the same as that of authentic 2,3,7,8-tetrachlorodibenzo-p-dioxin. This means that it is likely that the observed signals were from 2,3,7,8-TCDD, but this conclusion is not unambiguous.

Appendix I

A Report of Analyses by

Robert Harless, EPA,

of selected extracts

prepared by the

University of Nebraska group

Fractions of selected extracts were sealed in glass tubes and submitted to Robert Harless, U. S. Environmental Protection Agency, Mail Drop 69, Research Triangle Park, North Carolina 27711, for GC/MS analysis. Mr. Harless employs a high resolution capillary column GC separation and a medium resolution (R = 5000-6000) mass spectrometry analysis. Although he does not monitor the exact mass of TCDD by peak matching, he is able to separte more of the TCDD isomers than we at Nebraska.

His results are included in the following table. We are not reporting any percent recovery because of the difficulty in reliable handling of the trace levels of extracts. For positive detections, the retention time is the same as for 2,3,7, 8-TCDD. Our concentrations are also supplied for ease in making comparisons.

Table 2. Analysis of TCDD in samples of human fat extracted at UN-L.

Our Code	VA Code	Conc (ppt)[1]	Detection Limit	Conc. (UN-L)
17-NR-01	21	3	0.7	nd[3]
17-NR-02	18	5	0.8	nd
17-NR-03	13	nd	0.2	nd
17-NR-04	10	36	3.0	23
17-NR-05	9	3	1.0	nd
17-NR-06	8	3	0.6	nd[3]
17-NR-07	3	3	1.0	nd[3]
17-NR-08[2]	-	4.5	0.7	9

1. Parts-per-trillion
2. Quality Control Sample. True value = 9 parts-per-trillion
3. Signal at m/e 322 with the exact mass of TCDD, however, the isotope ratio ($i_{320/322}$) is incorrect.