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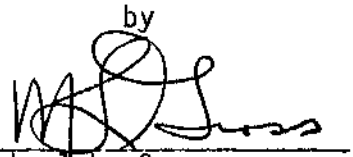
Report to

Veterans Administration

Dr. Lyndon Lee

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

by



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Professor and Director

12-3-79

Date

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

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

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).

Mass Spectrometer Conditions

The electron impact source was used at 70eV ionizing energy and an accelerating voltage of 8KV. The source was set at 260°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 3000Hz 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 0.77 (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 is adjusted to 100% recovery.

Validation of TCDD is considered acceptable if the observed ratio of signals is 9.77 $\pm$ 0.10.

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.

Interpretation of Data (Tentative)

As has been reported previously, a tetrachlorodibenzo-p-dioxin has been detected in a number of the human fat samples analyzed. Each of these detections have been validated by reanalyzing the extract as explained in the section on GC/HRMS Analysis. We do not consider any sample to be definitely positive unless it shows detected signals in both the original analysis and in the validation study.

The validation study, therefore, adds confidence to our assignment of positive detections. However, it does rule out contamination of the sample in our laboratory or by the surgeons who have removed the tissue. The former can be dismissed by employing the "method validation" approach developed during our collaboration with the Environmental Protection Agency (EPA). This procedure has been implemented by sending to EPA scientists portions of eight samples. These samples have been coded differently, and the EPA scientists are not aware of our results. In addition, suitable "spiked" controls have been included.

If similar results are obtained by the EPA, then there is high confidence that TCDD indeed exists in these specimens. The total analytical approach will involve, at least for selection of samples, extraction in two different laboratories and analysis in two different laboratories. The analytical procedures employed in the GC/MS are different but complementary. Our laboratory workers use packed column GC and high resolution peak matching mass spectrometry. The latter enables us to measure the exact mass of the eluting material to within 5 parts-per-million. The EPA laboratory uses high resolution (capillary column) GC and lower resolving power mass spectrometry. Good agreement among both of these approaches will allow us to extrapolate certainty to the remaining samples not submitted to the "method validation" approach.

Status of the Project

Twenty-two samples and controls have been analyzed at the Midwest Center for Mass Spectrometry, and the selected samples submitted to the EPA. ~~They have analyzed the samples once and found nearly all to be positive at levels considerably higher than we did. This indicated that very low level contamination exists in their laboratory~~. All analyses are now being repeated.

We have analyzed all samples and reported the data (preliminary) to the VA. However, because of the large numbers of positive detections in the last set (final ten samples), we have reextracted the material and are waiting to reanalyze them by GC/MS. Instrument problems have temporarily reduced ~~to sensivity~~ the sensitivity of our GC/MS and reanalysis will not be attempted until this problem is resolved.