The Determination of Pentachlorophenol and Hexachlorophene in Human Adipose Tissue

by
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The identification and quantitation of potential chemical pollutants and their metabolites in human adipose tissue are of great importance for monitoring human exposure to these environmental compounds. The two highly chlorinated phenols, pentachlorophenol (PCP) and hexachlorophene [2,2-methylenebis (3,4 6-trichlorophenol] (HCP), are widely used, and it will be of great interest to establish the extent of their storage in human adipose tissue.

The toxicological importance of PCP has been described by BEVENUE and BECKMAN (1967) and that of HCP by GUMP (1969), KIMBROUGH (1971) and KIMBROUGH and GAINES (1971). Adequate methods of analysis in urine and several tissues where relatively high levels of these compounds are present by virtue of acute high exposure have been developed, and urinary excretion rates have been established.

Gas chromatographic analysis of PCP in human urine and blood after formation of the alkyl ethers was reported by CRANMER and FREAL (1970), BEVENUE, et al (1968), and RIVERS (1971). BARTHEL et al (1969) described a method for the determination of PCP in blood, urine, tissue and clothing in which the underivatized PCP was injected on a gas chromatographic column containing 3% diethylene glycol succinate (DEGS) plus 2% syrupy phosphoric acid.

Electron capture gas chromatography was used to determine HCP in urine, blood, tissues and agricultural samples after methylation to produce the corresponding dimethoxy ether [2,2' methylenebis (3,4,6-trichloromethoxybenzene)] (CURLEY and HAWK, 1971, and GUTENMANN and LISK, 1970). PORCARO and SHUBIAK (1968) PORCARO et al. (1969) determined HCP on human skin and blood by gas chromatography after preparing the silyl ether. BACHMANN and SHETLAR (1969) determined HCP in biological tissue and fluids by electron capture gas chromatography without preparing a derivative. The purpose of this investigation was to develop a method

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for the determination of low levels of PCP and HCP in human adipose tissue of the general population.

EXPERIMENTAL

Gas chromatograph, Micro-Tek MT-220 equipped with tritium foil electron capture detector, and two U-shaped glass columns: a) For HCP: 2' x 1/4" o.d., packed with 60-80 mesh Gas Chrom Q coated with 2% SE-30; nitrogen flow rate 100 ml/min. b) For PCP: 6' x 1/4" o.d., packed with 80/100 mesh Chromosorb W. HP coated with 4% SE-30 + 6% QF-1; nitrogen flow rate 60 ml/min. Temperatures: Inlet, 230°C; columns, 200°C; detector, 215°C; transfer line, 240°C.

Tissue grinder, Duall Kontes #885380; Chromaflex column, size 22, Kontes #420100; Centrifuge tubes, 13-ml glass stoppered and graduated; Concentrator tubes, 10-ml. Kontes 570050; Disposable pipets; centrifuge.

Reagents

Sodium hydroxide AR; Potassium hydroxide AR; Hydrochloric acid AR; diethyl ether AR; Benzene and hexane nanograde; N-ethyl-N'-nitro-N-nitrosoguanidine, Aldrich Chemical Co.; Sodium sulfate anhydrous extracted with benzene in a Soxhlet extractor; Silica gel, Woelm, activity grade I; distilled water extracted with benzene.

Preparation of Diazoethane Reagent

Prepare the reagent using the method of STANLEY (1966). The reagent should be prepared daily and stored in a freezer. The adherence to the manufacturer's instructions for handling the reagent is recommended.

Preparation of Silica Gel Microcolumn:

Dry the silica gel for 48 hours at 170°C and store in an oven maintained at 130°C. On the day of use, remove the adsorbent from the 130°C oven to a desiccator and allow to come to room temperature. Weigh 10 grams of the dry silica gel into a vial with a Teflon-lined screw cap, add 0.10 ml water and immediately cover the container tightly and mix. Allow the adsorbent to equilibrate for 2-3 hours with intermittent mixing. No particles should adhere to the sides of the vial after equilibration. The standardized silica gel will contain approximately 1% water.

Just prior to use, prepare the chromatographic columns by adding 1 gram of silica gel (1% water) to the column which has been plugged with a small, loose wad of glass wool. Add 2 g. of anhydrous sodium sulfate. Introduce the derivatized PCP and HCP in hexane onto the column.

Preparation of Standard Solutions:

a) PCP: Prepare hexane solutions at concentrations of 2,4, and 20 ng/0.1 ml.

b) HCP: Prepare hexane solutions at concentrations of 2,20 and 200 ng/0.1 ml.

Preparation of Standard Curves:

Prepare mixtures of PCP and HCP as listed below by pipetting 0.1 ml aliquots of the appropriate individual standard solutions into 13-ml centrifuge tubes.

1) 2 ng PCP + 2 ng HCP

2) 4 ng PCP + 20 ng HCP

3) 20 ng PCP + 200 ng HCP

To each tube add 1.5 ml hexane and 0.5 ml 10% NaOH solution. Mix on a Vortex mixer for 1 min. and centrifuge to separate the two layers. Aspirate the hexane layer and discard. Add another 1.5 ml hexane, mix, centrifuge and discard the hexane layer. Repeat the hexane extraction once more. Add 0.5 ml concentrated HCl to the aqueous solution, mix and allow to cool to room temperature. Add 1.5 ml diethyl ether, mix on a Vortex mixer for 30 seconds and allow the two layers to separate. Transfer the ether layer to a 13-ml centrifuge tube with a disposable pipet. Repeat the ether extraction twice, transferring each ether extract to the 13-ml centrifuge tube. In a well ventillated hood, add 2 ml of freshly prepared dizaoethane and allow to stand for 20 minutes. Remove excess reagent by bubbling dry nitrogen through the solution, using a disposable pipet for delivery. Continue bubbling nitrogen until the solution has evaporated. Add 1 ml hexane and 0.5 g anhydrous sodium sulfate, and mix for a few seconds on the Vortex mixer. Using the nitrogen delivery pipet, transfer the hexane extract containing ethylated PCP and HCP to the silica gel microcolumn.

Rinse the centrifuge tube with 1 ml hexane and transfer to

the column. Repeat the hexane rinse and transfer to the column once more. Allow the hexane to sink into the column and discard that eluate. Add 8 ml 10% benzene in hexane to the column and collect this fraction, which contains ethylated PCP. As soon as the solvent sinks in the column, change collection tubes and continue eluting with 8 ml 60% benzene in hexane. This fraction contains ethylated HCP.

Adjust the volume of the first fraction (10% benzene in hexane) if necessary to exactly 8 ml with hexane. Inject 10 µl from each of the three concentrations of PCP into the gas chromatograph. The sensitivity of the electron capture detector to PCP derivative is approximately 2.5 pg.

Concentrate the second fraction (60% benzene in hexane) to a volume of 0.5 ml under a gentle stream of nitrogen. Inject 10 µl from each of the three concentrations of HCP into the gas chromatograph. The sensitivity of the electron capture detector to ethylated HCP is 40 pg. A complete reagent blank must be run through the entire procedure with each set of samples and standards.

Analysis of Adipose Tissue:

Accurately weigh approximately 200 mg of adipose tissue directly into a Duall tissue grinder. Add 1 ml of hexane, mix on Vortex mixer, and allow to stand for about 30 min. Grind the sample, remove the pestle and wash it with 0.5 ml hexane, allowing the hexane wash to drain into the grinding tube. Add 0.5 ml 10% NaOH, proceed with analysis as described in the preparation of standard curves beginning with "Mix on Vortex mixer for 1 min...."

RESULTS AND DISCUSSION

The ethylated derivatives of PCP and HCP were selected over the methylated because of their better gas chromatographic properties. The limits of detectability for PCP and HCP in adipose tissue are 5 and 10 ppb, respectively. Typical chromatograms of PCP and HCP in human fat at the reported levels of detectability are shown in Figure 1. The background from the reagent blank did not allow the detection of lower levels of PCP and HCP.

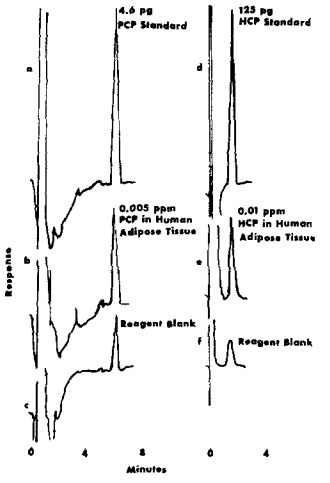

Fig. 1 Chromatograms of Ethlyated PCP and HCP
a Standard PCP 4.6 pg.
b 0.005 ppm PCP in Human Adipose Tissue
c Reagent Blank - PCP
d Standard HCP 125 pg.
e 0.01 ppm HCP in Human Adipose Tissue
f Reagent Blank - HCP

To establish recovery of both PCP and HCP from human adipose tissue, a pooled sample was prepared by rendering adipose tissue from several individuals at 60°C in a water bath for about two hours. The melt was filtered through glass wool to remove any connective or muscle tissues. The sample of pooled and rendered fat was prepared in order to eliminate sample variations. The sample was kept in a freezer to minimize possible loss or deterioration.

Samples from the pooled human fat weighing 100-300 mg were analyzed for both PCP and HCP using the procedure described in the analysis of adipose tissue. An average of 5 ppb PCP was found in six replicates and 30 ppb HCP in 10 replicates of human adipose tissue. Replicate samples of the pooled fat (100-300 mg) were spiked with 4.6 ng PCP and 10 ng HCP. The average recoveries of PCP and HCP from the replicates analyzed were 75% and 96%, respectively.

Table 1 illustrates the levels of PCP and HCP in human adipose tissue samples from the general population. The HCP content ranged from N.D. to 80 ppb with a mean value of 20 ppb, if the high HCP content of sample 3 is not included.

TABLE 1

Sample No.	PCP	HCP	Sample No.	PCP	HCP
1	19	20	11	22	ND
2	17	30	12	14	30
3	52	1000	13	35	20
4	45	30	14	52	ND
5	23	20	15	24	ND
6	25	20	16	12	10
7	21	10	17	12	10
8	35	ND	18	29	ND
9	17	10	19*	5	30
10	20	80			

* Pooled and rendered human fat sample.
ND = Not Detectable.

It can be concluded on the basis of the analysis of human adipose tissue samples that humans are continuously exposed to low levels of PCP and HCP from the environment, food supplies, and disinfectants. The possibility of toxicological effects from continued exposure of animals and humans to PCP and HCP at high and low levels has been considered, but to date there are no available studies to establish the "no effect" level of these toxicants. The procedure described in this paper is being made available to the pharmacologists and toxicologists to aid them in their studies.

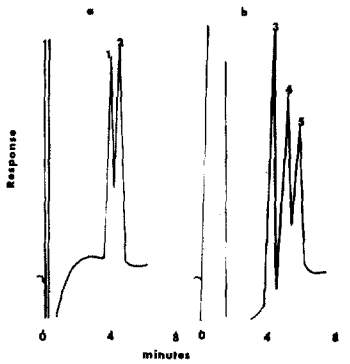

Fig. 2 Chromatograms of Various Derivatives of HCP
a G.C. column 2' x ¼" SE-30 2% Nitrogen flow rate 100 ml/min, oven temp. 200°C, tritium detector
1 - methyl derivative
2 - ethyl derivative
b. G.C. column 2' x ¼" OV-101 20% Nitrogen flow rate 100 ml./min., oven temp. 270°C nickel detector
3 - ethyl derivative
4 - trimethylsilyl derivative
5 - chloroacetate derivative

In addition to the sensitivity of the method for the determination of low levels of PCP and HCP in fat, it has an inherent advantage in its ability to provide confirmation of identity. The use of aqueous sodium hydroxide and the extraction of this basic solution with hexane to remove intact chlorinated hydrocarbons which interfere with the determination confirms that the compounds remaining in the alkali media are acidic compounds which form sodium salts upon treatment with a base. The preparation of other derivatives of PCP and HCP can also be used as further confirmation of identity. CRANMER and FREAL (1970) prepared the methyl, ethyl, propyl, isobutyl, butyl, isoamyl, amyl ethers of PCP, which have different retentions when injected onto various gas chromatographic columns. The methyl derivative (CURLEY and HAWK, 1971), ethyl and silyl derivatives (PORCARO and SHUBIAK, 1968, PORCARO et al, 1969) and chloroacetate derivative (SHAFIK et al, 1971) of HCP are also separated by gas chromatography. Figure 2 illustrates chromatograms of various derivatives of HCP.

Silica gel column chromatography employing selective differential elution of PCP with 10% benzene in hexane and HCP with 60% benzene in hexane is a further means of confirmation.

Confirmation of the diethyl ether of hexachlorophene and the ethyl ether of pentachlorophenol was accomplished by mass spectral direct probe analysis.

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