A Study of the Inhalation of Pentachlorophenol by Rats
Part I
A Method for the Determination of Pentachlorophenol in Rat Plasma, Urine and Tissue and in Aerosol Samples

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Pentachlorophenol (PCP) is a widely used Pesticide. In agriculture it is used as a defoliant, herbicide and insecticide and in industry and households for the treatment of wood against termites and as a preservative of various products which are prone to microbiological attacks.

Like so many other chlorinated organic pesticides, PCP has become a relatively long term contaminant of the environment. It has been found in wells, rivers and in the urine of every person now being examined (BEVENUE, et al., 1967).

PCP, although moderate in toxicity, can be hazardous as demonstrated by cases where death resulted from intense exposure (BERGNER, et al., 1965) (MASON, et al., 1965) (SMITH, et al., 1967).

Several studies have been done relative to the dermal and gastric toxicity of the compound (DEICHMANN, et al., 1942) (WALTERS, 1952) (STOHLMAN, 1951), but nothing has been reported with respect to potential inhalation problems. Air concentrations of PCP have been determined in treated buildings and in areas of liquid applications (CASARETT, et al., 1969). This is not surprising since PCP has an appreciable vapor pressure. This suggests an alternate route of absorption, that of inhalation. To investigate the effect of PCP administered in this manner, an exposure study was planned using small laboratory animals. This required the analysis of numerous tissue, blood, and urine and aerosol samples. Existing analytical procedures were modified to suit these special needs until a simple method was developed for the rapid determination of PCP in these different samples.

INSTRUMENTS AND MATERIALS

Metabolic collection cages for rats
Fischer Roto Rack

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Bulletin of Environmental Contamination & Toxicology,
Vol. 15, No. 1 © 1976 by Springer-Verlag New York Inc.

Gas chromatograph: Bendix 2500 with Tracor Ni63 electron capture detector
Inlet temperature 235°C, detector 280°C, oven 170°C
nitrogen flow 75 ml/min
Gas chromatographic columns: U-shaped glass 6' x ¼" packed with 1.5% OV-17 + 1.95% QF1 100/200 mesh AW Treatment (Applied Science Laboratories)
Tissue grinders, Potter Elvehjem size 24 (Kontes K-885500)
30 ml impingers, chromatoglex columns 9 mm Kontes K-420160. (The columns were packed with 10 cm florosil and 2 cm of anhydrous sodium sulfate).

REAGENTS AND SOLUTIONS

Isopropanol, sulfuric acid, potassium hydroxide, sodium sulfate, anhydrous (Mallinckrodt reagent grade)
Hexane, benzene, isooctane, ethyl ether (Mallinckrodt nanograde)
Tetrachlorohydroquinone (Eastman Kodak Co. No. 2280)
Pentachlorophenol (Eastman Kodak Co. No. 3462)
Diazomethane and diazoethane were prepared from N-methyl-N'-Nitro-N-Nitrosoguadinine 97% and N-ethyl-N'-Nitro-N-Nitrosoguadinine according to the direction of the manufacturer (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin)
Extracted water: distilled water was acidified extracted with hexane 10 to 1 - and neutralized with KOH
Florosil, PR grade (Floridin Company, Berkeley Springs, W. Va.)
Absolute standard: A stock solution was prepared containing 100 mg PCP in 100 ml isooctane. Standard solutions of methylated and ethylated PCP were made which contained 20 pg PCP per µl. Absolute standards were also prepared from methylated and ethylated Tetrachlorohydroquinone derivative.
Extracted Standard: Another stock solution contained 100 mg PCP in 100 ml of 0.1 N potassium hydroxide. An aliquot of this solution was diluted with 0.1 N potassium hydroxide to made a solution containing 500 ng PCP per ml.
One ml was pipetted into a 20 ml culture tube with a teflon lined screw cap. 4 ml extracted water and 5 ml hexane were added. The sample was then treated like the tissue samples. When finally diluted to 25 ml it contained 20 pg PCP per µl and was used as a standard against which all other samples were measured. The absolute standard was a control for the extracted standard.

DETERMINATION OF PENTACHLOROPHENOL IN TISSUE, PLASMA, AND URINE

Sampling

A. Blood: The blood was removed from the inferior vena cava of the anesthesized animals with heparinized 10 cc syringe equipped with a 21 gauge needle and centrifuged shortly thereafter. The plasma was pipetted into test tubes which were capped and stored in the refrigerator.

B. Tissue: The whole organs were removed after the animal had been exsanguinated and transferred into saline filled petrie dishes. After the excess blood had been removed, they were dried on gauze, weighed, and placed into tissue grinders. It was helpful to cut the tissue into small pieces before homogenization was attempted. 5 N KOH was added in a ratio of ca. 1-2 ml per gram of tissue to aid disintegration of the various tissues. For lung samples 2 ml was used, for liver samples 10 ml. Little pieces which clung to the walls of the grinders were washed down with 1 N KOH which was used sparingly. The grinders were then capped with parafilm and stored for a few days in a dark place at room temperature.

After the tissue had sufficiently disintegrated, which took at least one day, each tissue sample was homogenized with a pestle and transferred to a graduated test tube. The tissue grinder and the pestle were washed with extracted water several times until no residue remained. All the washings were added to the sample in the test tube and the final volume was adjusted according to the specific tissue: lung 15 ml, liver 50 ml. These homogenates were stored in the refrigerator until analyzed.

C. Urine: The urine was collected in 25 ml graduated cylinders which were placed under the collection cages. The volumes were measured and recorded daily. The funnel part of the collecting cage was rinsed with 10 ml of 0.1 N KOH and the rinse was combined with the urine sample, transferred to a 100 ml volumetric flask and stored in the refrigerator.

D. Air-aerosol Samples: The air-aerosol mixture was collected in a 25 ml impinger which contained 15 ml of a 0.1 N KOH solution. A second impinger was attached to the first one which in turn was connected to a flow meter. The air-aerosol mixture was pumped through the impingers at a rate of 0.5 liter/min for the duration of the exposure which varied according to the type of experiment. The liquid from both impingers was transferred into a 100 ml flask. The impingers were rinsed three times with a 0.1 N KOH solution which was added to the sample.

EXTRACTION

A. Tissue: The homogenate was removed from the refrigerator, equilibrated to room temperature, mixed thoroughly, and then an aliquot was pipetted into a 20 ml screw capped culture tube. For liver, 1-2 ml out of 50 ml was the usual sample size; the lung sample analyzed was between 1 and 5 ml of a 15 ml total. The volume of the sample was increased to 5 ml by adding extracted water to the tube. The sample was then frozen or partially frozen. 5 ml hexane was added and the sample was then acidified with 0.5 ml conc H_2SO_4. When all the ice had dissolved and the sample had reached room temperature, it was extracted for 2 minutes with the hexane on the vortex mixer. Care had to be taken that the mixture remained in a spinning motion throughout this time. If this could not be done, the sample was shaken manually for 2 minutes or it was spun on the Roto Rack for 30 minutes at

60 r.p.m. The resulting emulsion was spun down by centrifuging for 10 minutes at ca. 4000 r.p.m. Often a repeated centrifuging was necessary to break a persistent emulsion. When a distinct separation of the two phases had been reached, the hexane layer was pipetted into a 25 ml volumetric flask. The extraction procedure was repeated twice and the combined extracts were collected in the same flask.

B. Plasma: Usually 0.5 ml of sample was increased to 4.5 ml with extracted water. 5 ml of benzene was added and the sample was acidified with 0.5 ml 1 N sulfuric acid. There were four benzene extractions which were done in the same manner as the hexane extractions described for the tissue samples.

C. Urine: Of 100 ml an aliquot of 1 ml was extracted with hexane like the tissue samples.

D. Air-aerosol: Of 100 ml, 1 ml was extracted with hexane (as with the tissue).

ALKYLATION

An aliquot which was expected to contain not more than 1 µg PCP was pipetted into a 15 ml graduated test tube, placed into a lukewarm waterbath and condensed under a nitrogen stream to a volume of 2 ml. About 0.5 ml of the alkylating reagent in ethyl ether was added. All urine samples was ethylated, all other samples were methylated. The solution of diazoalkane plus hexane or benzene extract was mixed and allowed to react. After an interval of at least 30 minutes the volume of the sample was condense to 1 ml by using the same method as described before. This removed the excess diazoalkane (the yellow color disappeared) and reduced the sample to a size which was suitable for the following purification procedure.

PURIFICATION

All samples - especially the tissue samples - contained compounds which interfered with the gas chromatographic determination. To remove these impurities, each sample was transferred to a chromatoflex florosil column which had previously been rinsed with 10 ml hexane. The sample was eluted with 10 ml hexane and collected in a 25 ml flask. The extract was now ready to be injected into the gas chromatograph.

GAS CHROMATOGRAPHIC DETERMINATION

The electrometer of the electron capture gas chromatograph was adjusted to an input attenuation of 10 and a recorder attenuation of 200. The detector voltage was adjusted to about 90% of the plateau to give a linear range between 20 and 120

picograms. An aliquot of the final extract was injected and compared to similarly sized peaks of standard PCP ethers. Dilutions or concentrations of the extract were made when necessary to keep the injected amount within the linear range of the detector.

RESULTS

The gas chromatograms of the PCP residues in the various samples showed no interfering compounds. The baselines were even cleaner than those produced by the absolute standard. All results were easily duplicated within standard error of 3%. The lower limit of detectability was 20 parts per billion.

Table I, II, and III show the analytical reproducibility and recovery of PCP from liver, lung, plasma and urine samples. Each sample originated from the same solution or homogenate which was fortified with the same amount of PCP dissolved in a 0.01 N sodium hydroxide solution. The aerosol samples are not included in the following tables as they consist only of PCP in a sodium hydroxide solution like the extracted standard.

TABLE I

Recovery and Reproducibility of Pentachlorophenol from Tissue Samples

µg PCP Found in 1 ml of Homogenate	µg PCP Added	µg PCP Recovered	% Recovery
	a) Liver		
0.0625	2.500	2.384	93.0
0.0625	2.500	2.384	93.0
0.0625	2.500	2.325	90.7
0.0625	2.500	2.325	90.7
0.0625	2.500	2.296	89.6
Average:	91.4%	Std. error:	0.68%
	b) Lung		
0.047	2.500	2.343	91.2
0.047	2.500	2.312	90.8
0.047	2.500	2.237	87.8
0.047	2.500	2.531	99.4
0.047	2.500	2.434	95.6
Average:	93.0%	Std. error:	2.04%

TABLE II

Recovery and Reproducibility of Pentachlorophenol from Plasma

µg PCP Found in 0.5 ml of Plasma	µg PCP Added	µg PCP Recovered	% Recovery
0.056	2.500	2.465	96.4
0.056	2.500	2.375	92.9
0.056	2.500	2.404	94.0
0.056	2.500	2.512	98.2
0.056	2.500	2.339	91.5

Average: 94.6% Std. error: 1.20%

TABLE III

Recovery and Reproducibility of Pentachlorophenol from Urine

µg PCP Found in 1.0 ml of Urine	µg PCP Added	µg PCP Recovered	% Recovery
0.043	2.500	2.410	94.7
0.043	2.500	2.336	91.8
0.043	2.500	2.446	96.2
0.043	2.500	2.517	98.9
0.043	2.500	2.410	94.7

Average: 95.3% Std. error: 1.16%

DISCUSSION

Essentially the modifications were a reduction of initial sample size, adaptation to tissue types and incorporation of a florosil clean up step to prevent contamination of the GC detector by co-extracted products. Neither recovery nor accuracy have been sacrificed by these modifications, and analysis time per sample has been considerably reduced. Confirmation was accomplished by mass spectroscopy.

Initially all samples were methylated, however, urine samples having the highest concentration of PCP, showed some interference in the chromatograms as evidenced by unsymmetrical peaks. This interference was due to the methylated metabolic product, tetrachlorohydroquinone (TCHQ), as confirmed my mass spectroscopy. Ethylation of the urine extracts accomplished separation of this product from the parent compound under the GC conditions used. All samples agreed with the standards in regard to their retention

time which was 0.45 for the methylated PCP and 0.47 for the ethylated compound. The ethylated TCHQ derivative had a relative retention time of 0.59.

Hexane was chosen for most of the extractions because it was almost as efficient as benzene with the advantage of a lower toxicity. However, hexane extractions of plasma gave a very poor yield which was probably due to the protein binding properties of PCP. This assumption is supported by a blood plasma protein binding study which has been conducted in this laboratory and is shown in Part V of this study. When benzene was used, a satisfactory recovery could be achieved in four extractions. The strong emulsions which formed during the extraction could be partially controlled by acidifying the sample with not much more 1 N H_2SO_4 than needed to insure complete conversion of any pentachlorophenate in the plasma to pentachlorophenol and by adding a few drops of isopropanol to the sample after the centrifugation.

It was found, as reported by CRENMER and FREAL (1970) that one single hexane extraction of acidified urine yields 90% of the PCP present. This, however, is only true with samples containing not much more PCP than found in the urine of the average population. Under experimental conditions, where the PCP level in urine is higher by a factor of 1000, three extractions were found necessary.

The tissue samples were frozen solid prior to the acidification to avoid superheating. It was found that the heat resulting from the reaction of the concentrated H_2SO_4 with the highly alkaline homogenate produced various compounds which greatly disturbed the chromatogram and contaminated the detector and gave interfering peaks on the chromatogram. The freezing method helped to keep the extract free from most impurities. A subsequent purification with a florosil column which had been heat treated for 24 hours at 135°C yielded samples which were as clean as the standards. The urine and plasma extracts contained only few interfering substances which were removed with florosil columns which did not have to be activated.

SUMMARY

Simple and flexible methods have been designed for the determination of pentachlorophenol in animal tissue, blood plasma, urine, and aerosol. The isolation of the pesticide is achieved through its extraction with benzene or hexane after acidification, derivatization, and its subsequent purification through florosil columns. A brief description of the sampling methods is included.

The described procedures are especially suited for exposure experiments where different types of samples with a large variety of PCP concentrations are encountered.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Ted Norton for his valuable suggestions in reviewing this paper and Mr. Gary Mols for his technical assistance.

This work was supported by a grant awarded by the National Institute of Health.

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