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Determination of Pentachlorophenol in Urine: The Importance of Hydrolysis

A gas chromatographic method for more reliable determination of pentachlorophenol (PCP) in urine has been developed. After hydrolysis and extraction the sample was reacted with diazomethane to produce the methyl ether of PCP prior to analysis by electron-capture gas chromatography. An acid alumina column clean-up system was developed to remove interferences from the sample extracts and allow detectability of 1 ppb PCP. Average recoveries of greater than 90% were obtained from urine fortified with known amounts of PCP. The importance of hydrolysis and comparisons of present methodologies will also be presented.

Pentachlorophenol (PCP) is a well-known, extensively used pesticide and a discussion on its uses, toxicity, and fate in the environment is found in the literature (Bevenue and Beckman, 1967). The occurrence of PCP in human urine is well documented (Bevenue et al., 1967; Wyllie et al., 1975).

Several methods for the determination of PCP in urine have been reported in the literature. Bevenue et al. (1966) and Rivers (1972) acidified urine, extracted with organic solvent, and methylated the urine extract with diazomethane prior to electron-capture gas chromatography (EC-GC). Cranmer and Freal (1970) partitioned possible interfering compounds into base prior to acidification and extraction of PCP into organic solvent. The urine extract was methylated with diazomethane and analyzed by EC-GC. Shafik et al. (1973) determined PCP and other halo- and nitrophenols in urine by acid reflux, extraction with ethyl ether, derivatization with diazoethane, and separation on a silica gel column prior to EC-GC analysis.

Methodology in the literature does not include a hydrolysis procedure for the determination of PCP in urine. In our laboratory we found that hydrolysis gave a much higher level for biologically incorporated PCP than when other methods not employing hydrolysis were used. Because of this finding a highly selective and more quantitative method for the determination of PCP in urine at low parts per billion levels will be described.

MATERIALS AND METHODS

Apparatus. Tracor, MT-220, gas chromatograph equipped with a nickel-63 electron-capture detector was operated in the pulsed linearized mode. A Borosilicate glass column (1.8 m × 4 mm i.d.) was packed with 80/100 mesh Gas-Chrom Q coated with 5% OV-210. The column was operated at 160 °C with 5% methane in argon at a flow rate of 40 mL/min. Detector, inlet, and transfer line temperatures were 300, 235, and 220 °C, respectively. At 5×10^{-11} amps full scale, PCP methyl ether gave a half-scale deflection at 10 pg and has a relative retention ratio to aldrin of 0.49.

Reagents and Materials. Anhydrous, granular sodium sulfate was Soxhlet extracted for 4 h with hexane and oven-dried at 130 °C.

Acid alumina, Brockman Activity I (Fisher Scientific), was dried for 24 h at 130 °C and stored in a desiccator.

Potassium hydroxide and hydrochloric acid were reagent grade.

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine and pentachlorophenol (99+%) were obtained from Aldrich Chemical Co., Milwaukee, Wis. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine should be handled carefully since it is a known carcinogen.

All solvents were pesticide quality or equivalent.

Methylating Reagent. Potassium hydroxide (2.3 g) was dissolved in 2.3 mL of distilled water in a 125-mL Erlenmeyer flask and cooled to room temperature. Twenty-five milliliters of ethyl ether was then added and the flask was cooled in a refrigerator. The following step was carried out in a glovebox or a high-draft hood. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (1.5 g) was added in small portions to the flask with vigorous shaking. The ether layer was decanted into a scintillation vial and stored in a freezer (Stanley, 1966).

Preparation of Standard Solutions. An analytical standard of pentachlorophenol was prepared in acetone and stored at −15 °C in a brown glass bottle. A solution of PCP in acetone was methylated by adding 5 mL of diazomethane reagent in a high-draft hood (Howard and Yip, 1971). (CAUTION: Diazomethane is toxic and may be explosive under certain conditions.) The standard was

Table I. Recovery of PCP from Urine[a]

ppm added	% range	av % recov.	% rel SD[b]
1.0	95.2-97.8	96.5	±1.1
0.3	92.3-99.0	95.3	±2.8
0.1	93.0-95.0	94.1	±0.8
0.03	91.9-100.4	95.1	±3.7
0.01	90.6-96.3	93.2	±2.5
0.005	88.0-104.0	93.9	±7.1

[a] Four determinations. [b] SD, standard deviation.

allowed to stand for 1 h before evaporation of the ethyl ether, and either dilution or column cleanup prior to EC-GC determination.

Chromatography of PCP on Acid Alumina. A size 22-9 Chromaflex column (Kontes 420530) was loosely plugged with a small amount of glass wool. Acid alumina (4 g) was added in small increments with tapping. Anhydrous, granular Na_2SO_4 (1.6 g) was added to the top of the alumina. Thirty milliliters of 20% benzene in hexane was used to wash the column free of interferences. After thorough air-drying, the column was placed in an oven at 130 °C overnight prior to use.

A prepared column was removed from the oven and allowed to cool to room temperature. The column was wetted with 5 mL of hexane. When the solvent layer reached the top of the Na_2SO_4 an aliquot of 0.2 to 0.3 mL methylated sample or methylated standard was placed on top of the column. Quantitative transfer of the sample or standard was accomplished by three 0.5-mL rinsings with hexane. An additional 3.5 mL of hexane was added and the hexane fraction (5.0 mL) was collected and discarded. The pentachlorophenol methyl ether was eluted with 20 mL of 10% benzene in hexane and the volume adjusted prior to EC-GC analysis.

Analysis of Urine. Two milliliters of urine was transferred to a 20 × 125 mm Teflon-lined screw-cap culture tube and acidified with 0.5 mL of concentrated HCl. The tube was sealed and placed in a boiling water bath for 1 h with periodic shaking. The tube was removed and cooled to room temperature. The urine sample was extracted twice for 1 h each on a mechanical rotator at 30 to 50 rpm using two 5-mL portions of benzene. The sample was centrifuged after each extraction and the extracts combined in a 15-mL centrifuge tube. The benzene extracts were concentrated to a volume of 0.3 to 0.5 mL under a gentle stream of nitrogen in a water bath at 30 °C and methylated with 5 mL of diazomethane reagent. The methylated extract was allowed to stand for 1 h. Prior to column cleanup, the methylated urine extract was concentrated to approximately 0.3 mL under a gentle stream of nitrogen. Two milliliters of hexane was added and the sample was reconcentrated to a volume of 0.3 mL.

RESULTS AND DISCUSSION

As shown in Table I, recoveries of PCP from fortified urine averaged greater than 90% when corrected for background PCP. Method sensitivity is estimated to be 1 ppb for PCP in urine. Great difficulty was encountered in finding a control urine low enough in PCP to use for fortification purposes. A general population human urine with an average of 4 ppb PCP background was used for fortification purposes.

Column cleanup was found to be essential for the determination of PCP at levels below 30 ppb. Except for the method used by Shafik et al. (1973) none of the methods listed previously employ column cleanup. In our study, difficulty was encountered in determining low levels (30 ppb or less) of PCP in human urine using other methods.

Table II. Comparison of PCP Levels Found in Human Urine Samples by Different Methods

	PCP found, ppm[a]		
sample	EPA manual method[b]	method of Cranmer & Freal (1970)	present method
general population	<0.01	<0.01	0.02
general population	0.02	0.02	0.08
general population	0.01	<0.01	0.04
general population	<0.01	<0.01	0.02
exposed worker	0.54	0.21	3.68
exposed worker	0.41	0.31	1.71

[a] Average three determinations. [b] Combined method of Rivers (1972) and Cranmer and Freal (1970).

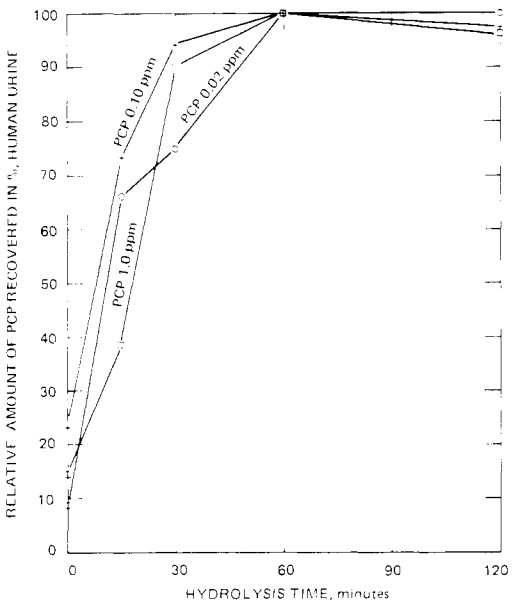

Figure 1. Relative amount of PCP found in human urine at various hydrolysis times.

Recoveries of less than 80% were noted for these methods at fortification levels of 30 ppb or less PCP. Column cleanup allows a detection limit of 1 ppb PCP in urine.

A comparison of PCP levels found in human urine samples by the EPA Analytical Manual Method (Thompson, 1974), the Cranmer and Freal (1970) method, and the method detailed in this paper is given in Table II. The method presented in this paper yields as much as a 17-fold higher PCP level after hydrolysis than do the other methods used for comparison.

To further substantiate the validity of this method, an experiment was performed to determine the recoverability of biologically incorporated PCP in urine for varying times of hydrolysis. Figure 1 illustrates the results of this experiment. PCP levels of 0.02, 0.10, and 1.00 ppm were found in three different unfortified human urine samples after 1 h of hydrolysis. With no hydrolysis of the urine samples (time 0), less than 25% of the recoverable PCP was obtained. A hydrolysis time of 1 h was necessary for the maximum freeing of presumable conjugated PCP in urine. Further hydrolysis did not yield additional PCP.

Bevenue et al. (1966) reported poor recoveries of PCP in urine when refluxed with sulfuric acid. Hydrolysis using "cold finger" condensers in a closed system gave a PCP value only slightly higher than when no hydrolysis was performed. Our study showed that when urine was hydrolyzed in a closed system that recoveries are higher than without hydrolysis.

The results presented in this paper serve to further demonstrate that simply fortifying samples in the laboratory does not always yield valid recovery data, particularly for biologically incorporated organic compounds. This is especially true for compounds of a polar nature which are usually excreted in urine as conjugates.

SUMMARY

Detailed methodology for the determination of pentachlorophenol in urine has been presented. A hydrolysis time of 1 h was determined to be essential for the determination of both free and conjugated PCP. An acid alumina column cleanup allowed levels of 1 ppb PCP to be detected quantitatively in urine. Recoveries of PCP from urine at fortification levels of 5 ppb and greater averaged better than 90%.

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Epimerization of α-(1-Carboxyethyl) Hydrogen Benzylpenicilloate

α-(1-Carboxyethyl) hydrogen benzylpenicilloate (III), a material formed by the interaction of lactic acid with benzylpenicillin (I) in meat, isomerizes when the meat is heated. The 5-epimer (IV) and, to a lesser extent, the 6-epimer (V) are formed.

In a study to determine the fate of benzylpenicillin (I) residues in meat, DePaolis et al. (1977) found that the major product formed upon freezer storage (−2 °C) was α-(1-carboxyethyl) hydrogen benzylpenicilloate (III, Figure 1). Cooking the meat resulted in the formation of larger amounts of III as well as formation of at least one of its isomers. This paper reports the efforts to isolate and determine the stereochemistry of the cooking product(s).

TLC analysis of the cooked meat extract revealed, in addition to benzylpenicilloic and benzylpenilloic acids, the presence of III at R_f 0.45 and a material at R_f 0.32. The quantity of material at R_f 0.32 increased at either higher temperature or with longer cooking times. Attempts to remove the material at R_f 0.32 with subsequent esterification by diazomethane were complicated by the fact that we could not completely rid the system of acetic acid, which had to be used in the eluant mixture and which preferentially reacted with diazomethane. Instead, the materials present in meat were extracted with methanol and eventually converted to their methyl esters with diazomethane as described by DePaolis et al. (1977). TLC separation of this mixture on silica gel (benzene–ethyl acetate, 1:1) gave, in addition to dimethyl benzylpenicilloate (R_f 0.62) and methyl benzylpenilloate isomers (R_f 0.28 and 0.18), material at R_fs 0.69 (dimethyl ester of III), 0.60, and 0.56. The latter two both gave essentially similar chemical ionization spectra as III, indicating that the three compounds were isomers. Under "normal" cooking conditions there appeared to be more R_f 0.60 material than material with R_f 0.56, making the former the compound of greater practical importance.

The same materials could be formed by heating III in an oil bath at 80–100 °C for 30 min. These conditions also promoted the conversion of benzylpenicilloic acid to benzylpenilloic acid. This was very helpful because dimethyl benzylpenicilloate had an R_f value very close to one of the materials of interest in our TLC solvent system.

Material III contains four assymetric carbon atoms, with epimerization most likely to occur at C-5 and C-6. Epimerization at C-6 in penicillins has been well documented (Kaiser and Kukolja, 1972). The different epimeric forms of benzylpenicilloates have been reported but only the stereochemistry of the epimer corresponding to naturally formed benzylpenicillin has been established with certainty (Sheehan and Cruickshank, 1956). For III, there are three other possible C-5, C-6 epimers (IV, V, and VI, Figure 1). One of these, V, was synthesized by reacting 6-*epi*-benzylpenicillin (II) with an equimolar amount of calcium lactate in dimethyl sulfoxide (Me_2SO) as described earlier (DePaolis et al., 1977) except that 100% Me_2SO was used instead of 50% Me_2SO–water. The change to 100% Me_2SO was necessitated by the fact that II reacted with calcium lactate at a slower rate than I. Conversion of the latter to II was achieved by treatment of I with trimethylchlorosilane (TMCS) and *N,O*-bis(trimethylsilyl)acetamide (BSA), followed by treatment with 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) as described by Vlietinck et al. (1973). The epimerization of benzylpenicillin to its 6-epimer could be monitored and quantitated by high-pressure liquid chromatography. The two

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