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A SIMPLE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF PENTA- AND TETRACHLOROPHENOLS IN URINE OF EXPOSED WORKERS

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SUMMARY

A liquid chromatographic method was developed for the simultaneous determination of penta- and tetrachlorophenols in urine. The method is more rapid than gas chromatographic methods and does not involve the use of such potentially dangerous compounds as benzene, diazomethane or pyridine, which have been used in several methods described previously.

INTRODUCTION

Chlorinated phenols and their sodium salts are used extensively as wood preservatives and pesticides. About 200,000 tonnes of chlorophenols are manufactured annually [1]. Technical chlorophenol products manufactured by chlorination of phenol vary in composition; those studied in this investigation contained 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol as their main components. Since chlorophenols are absorbed through the skin, occupational exposures have been monitored by measuring urinary excretion or blood levels rather than by measuring air concentrations [2—8]. Several sensitive gas chromatographic methods exist for the analysis of chlorophenols in biological fluids [3—20]; however, these involve extensive, time-consuming purification and derivatization steps. Some of these methods also require use of chemicals such as benzene, diazomethane and pyridine, which pose a health risk to the analyst. We describe a rapid and convenient liquid

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chromatographic (LC) method for the analysis of chlorophenols in the urine of exposed workers.

EXPERIMENTAL

Principle

After acid hydrolysis of conjugates, tetra- and pentachlorophenols are extracted in a mixture of *n*-hexane and isopropanol. The chlorophenols in the organic phase are quantitated by reversed-phase LC.

Equipment

The following liquid chromatographs were used: Spectra-Physics 3500B with "mixed-wavelength" (254 and 280 nm) absorbance detector, Model 230 and Rheodyne Injector No. 7120, or a Hewlett-Packard 1084B with 79875A variable-wavelength detector, 200—540 nm, with automatic sampling system 79842A. The columns used were: Spherisorb ODS, RP-18, 10 μm, 30 cm × 5 mm I.D. (Spectra-Physics, Santa Clara, CA, U.S.A.), Radial Pak A, RP-18, 10 μm, 10 cm × 8 mm I.D. (Waters Assoc., Milford, MA, U.S.A.), or Hewlett-Packard (Avondale, PA, U.S.A.) 79918B, RP-8, 10 μm.

Chemicals

The chlorophenols, 2,3,4,5-tetrachlorophenol (2,3,4,5-TCP) purum, 2,3,4,6-tetrachlorophenol (2,3,4,6-TCP) techn (with approx. 20% pentachlorophenol as contaminant), 2,3,5,6-tetrachlorophenol (2,3,5,6-TCP) purum, and pentachlorophenol (PCP) puriss, were purchased from Fluka (Buchs, Switzerland); hydrochloric acid pa., *n*-hexane rein, ammonium carbonate pa., and isopropanol pa., were purchased from Merck (Darmstadt, G.F.R.). The methanol was of high-pressure liquid chromatography grade and was purchased from Orion, Finland. Water was purified by double-distillation after deionization.

Procedure

Four millilitres of 6 mol/l hydrochloric acid were added to 8 ml of urine in a 25-ml test tube with a screw cap. After 60 min in a boiling-water bath, the tubes were cooled, and penta- and tetrachlorophenols were extracted in 8 ml of hexane—isopropanol (5:1, v/v) by shaking for 15 min in a mechanical shaker. After centrifugation, 5 ml of the organic phase were evaporated at 80°C to dryness in a covered water bath (there were holes in the cover for the tubes); the residue was dissolved in 0.5 ml of methanol—water (1:1, v/v) by vigorous shaking in a Rotamix test-tube shaker. Of this solution, 20—30 μl were injected into the liquid chromatograph. In the isocratic analysis, the mobile phase consisted of methanol and 0.05% ammonium carbonate in water; the optimal ratio of the two varied somewhat depending on the column, but was generally about 50% of methanol. The flow-rate was 2.0—3.0 ml/min. In separating different isomers of tetrachlorophenols, a linear gradient of 36 to 48% methanol within 12 min was used, after an isocratic elution at 36% for 10 min. The chlorophenols were detected with an ultraviolet detector at 254 nm. The standards, which consisted of urine from non-exposed persons and contained 0—15 μmol/l chlorophenols, were treated in the same way as the samples.

Quantitation was achieved by measurement of peak heights by the Spectra-Physics LC system, or by area integration with the Hewlett-Packard LC system.

RESULTS AND DISCUSSION

Hydrolysis of chlorophenol conjugates

Penta- and tetrachlorophenols occur in the urine of exposed workers partly as conjugates with glucuronic acid [20, 21], and such conjugates must be hydrolyzed before analysis. 2,3,4,6-Tetrachlorophenol conjugates are totally hydrolysed by about 2 mol/l hydrochloric acid at 100°C after 15 min; however, in confirmation of the findings of Edgerton and Moseman [9], 1 h was required to complete the hydrolysis of pentachlorophenol conjugates (Fig. 1).

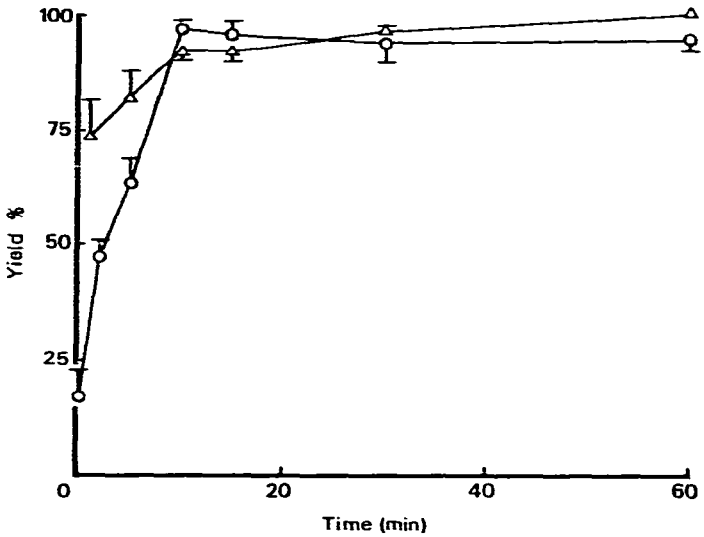


Fig. 1. Effect of hydrolysis time on the yield of 2,3,4,6-tetrachlorophenol (○) and pentachlorophenol (△) in urine. The points represent mean ± S.E.M. of three separate samples from workers exposed to chlorophenols.

The hydrolysis did not decrease the yield of added penta- and tetrachlorophenols (data not shown), indicating that the released aglycones are not destroyed by this procedure. It was shown earlier [3] that treatment with sulphuric acid, even at low temperatures, is enough to break conjugates of pentachlorophenol. We prefer to use hydrochloric acid, however, since sulphuric acid constitutes a health hazard in the laboratory, and since the addition of sulphuric acid to urine can easily cause overflow due to foaming of the mixture.

Extraction procedure

Chlorophenols are weak acids and can be extracted by organic solvents under acidic conditions. Various combinations of organic solvents have been used, including benzene [7, 9—13], hexane [14—16], diethyl ether [12, 18—20], isopropyl ether [8] and isopropanol [14—16]. In the present study a

mixture of hexane and isopropanol was used, which has the advantage that even when acidic urine is extracted no emulsion is formed.

Extraction efficiency for both 2,3,4,6-tetra- and pentachlorophenol is 85–87%. As chlorophenols have some volatility, there is, however, additional loss at 80°C in the evaporation. The final yield for 2,3,4,6-tetrachlorophenol after evaporation is 54.6 ± 2.0% (mean ± S.D. from six different concentrations between 1.7 and 17.3 mol/l) and for pentachlorophenol 83.3 ± 3.7% (mean ± S.D. from six different concentrations between 1.5 and 15.3 mol/l). Thus, although the yield was rather low, the reproducibility was good. A nitrogen evaporator and an aluminium heater block did not give as reproducible results as the covered water bath.

Chromatographic separation

Using gradient elution with 36 to 48% methanol in ammonium carbonate, after 10 min of isocratic elution at 36%, the three isomers of tetrachlorophenol could be separated from each other and from pentachlorophenol (Fig. 2). With this technique the analysis time was nearly 25 min. However, since no more than trace amounts of 2,3,4,5- and 2,3,5,6-tetrachlorophenols were

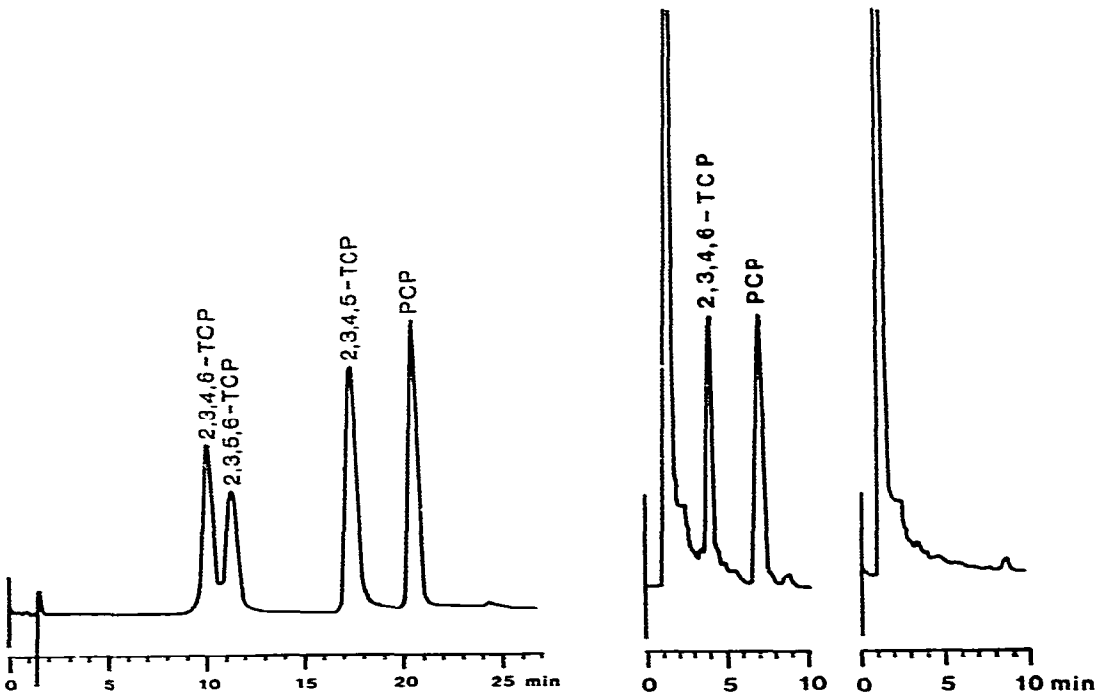


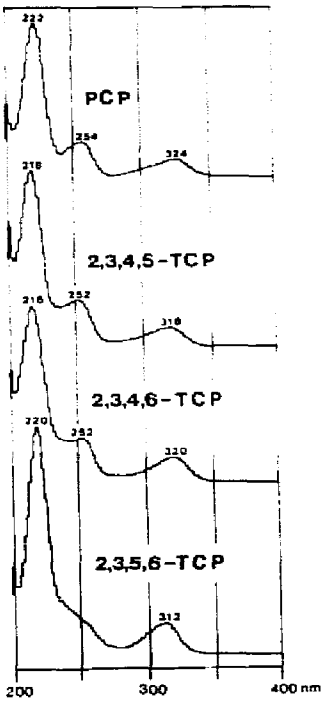
Fig. 2. Separation of tetrachlorophenol (TCP) isomers and pentachlorophenol (PCP) by the gradient elution technique; column; Radial Pak A (RP-18). Linear gradient of 36 to 48% methanol, after 10 min of isocratic elution at 36%. Ultraviolet detection at 254 nm.

Fig. 3. Left: separation of pentachlorophenol (PCP) (15 μmol/l) and 2,3,4,6-tetrachlorophenol (2,3,4,6-TCP) (17 μmol/l) added to urine of non-exposed persons. Right: tracing after no addition. Column, Radial Pak A (RP-18); elution with 53% methanol in 0.05% ammonium carbonate. Ultraviolet detection at 254 nm.

present in the urine of exposed workers, a simple isocratic analysis at about 50% methanol in ammonium carbonate was found to be generally sufficient (Fig. 3). In this way, an excellent separation of penta- and 2,3,4,6-tetrachlorophenol from each other and from interfering chemicals present in urine was achieved in less than 10 min.

Detection

Penta- and tetrachlorophenols have three distinct absorption maxima in the ultraviolet region (Fig. 4). The highest values were encountered at about 220 nm; however, that short wavelength is inconvenient, since many chemicals tend to interfere there. Efficient detection with low background was, however, achieved at 254 nm. It should be pointed out that the molar absorptivities of the tetrachlorophenol isomers differ and their quantitation may thus not depend on a common standard. At 254 nm, calibration graphs (prepared by adding known amounts of penta- and tetrachlorophenols to urine from non-exposed persons) were linear up to a concentration of at least 15 μmol/l. The regressions y (peak height) = 12.075x (concentration − 0.210 for pentachloro-



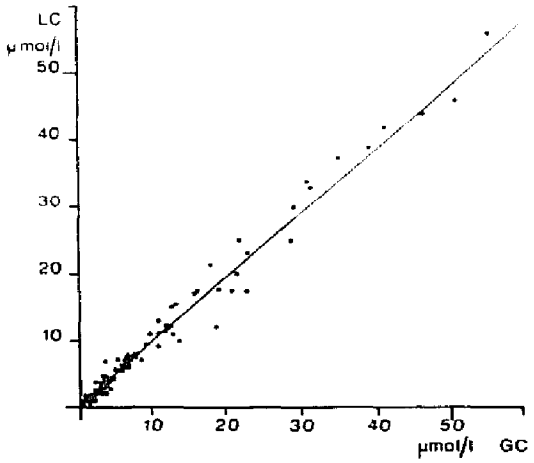


Fig. 4. Ultraviolet spectra of penta- (PCP) and tetrachlorophenol (2,3,4,5-TCP; 2,3,4,6-TCP; 2,3,5,6-TCP) standards, measured with a Hewlett-Packard 1084B liquid chromatograph equipped with a 79875A variable-wavelength detector, column RP-8 Hewlett-Packard 79918B, 10 μm; elution with 50% methanol in 0.05% ammonium carbonate.

Fig. 5. Comparison of the liquid (LC) and gas—liquid chromatographic (GC) analysis of 2,3,4,6-tetrachlorophenol. Calculations were based on 115 samples of 2,3,4,6-TCP; each point represents one or more equal results. The regression line y = 0.976 x + 0.034 (r = 0.988) is shown.

phenol and $y = 9.625x + 0.157$ for 2,3,4,6-tetrachlorophenol, had correlation coefficients in excess of 0.998.

Comparison with a gas—liquid chromatographic method

To further validate this LC procedure for the analysis of chlorophenols in urine, it was compared with a gas–liquid chromatographic method [15], based on Rudling's method [14], which makes use of hexane–isopropanol extraction, sodium tetraborate purification, acetylation and detection with electron capture. Fig. 5 shows that the two methods give similar results. The regression line had an equation of y (LC method) = $0.976\,x + 0.034$, and the correlation coefficient was 0.988.

Sensitivity and precision

The concentration of chlorophenol that gave rise to an absorption peak twice that of the background was 0.1 μmol/l for both penta- and 2,3,4,6-tetrachlorophenol when 8 ml of urine were used in the LC analysis. This sensitivity is lower than those of gas chromatographic methods: that of the gas chromato-

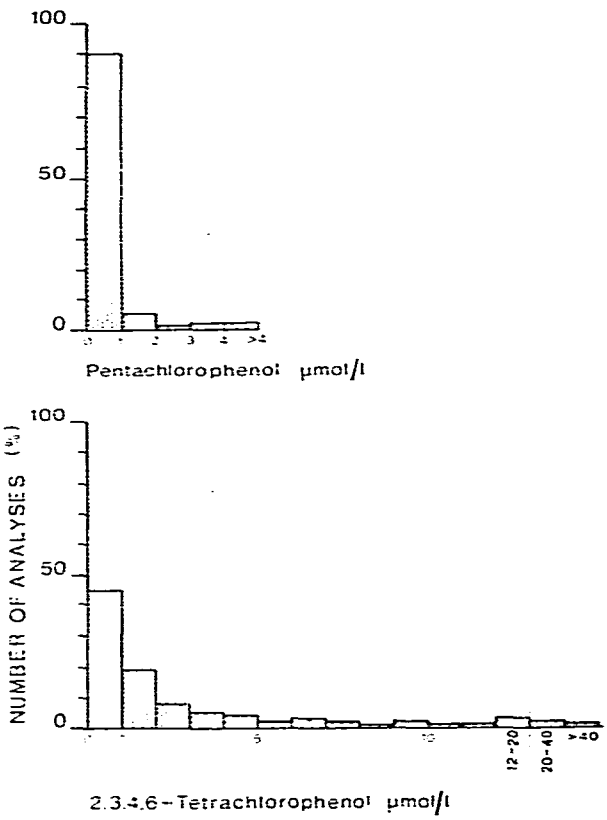


Fig. 6. The frequency distribution of the results of the analyses of penta- and 2,3,4,6-tetrachlorophenols in the urine of exposed workers in wood preservation and chlorophenol manufacture. During the years 1975—1976 the analysis was done gas chromatographically [15] (n=338), thereafter with the LC method (n=1038).

graphic method used for comparison in this study was 0.05 μmol/l with only 2 ml of urine used as the starting material. However, for biological monitoring of workers exposed to chlorophenols the sensitivity of the LC method presented is sufficient.

The coefficient of variation (between series, determined from ten separate preparations made during one day), was 2.6 and 2.5% for pentachlorophenol and 2,3,4,6-tetrachlorophenol, respectively, at concentrations of 12—13 μmol/l.

Application

The method described for the analysis of penta- and tetrachlorophenols was used for three years in Finland in the biological monitoring of workers exposed to chlorophenols during preservation of wood and manufacture of chlorophenols. The frequency distribution of the results is depictedin Fig. 6, showing that 90% of the workers excreted less than 1 μmol/l penta- and less than 9 μmol/l 2,3,4,6-tetrachlorophenol in the urine. In nearly all cases 2,3,4,6-tetrachlorophenol was the prevailing chlorophenol in the urine. The exposure of these workers was thus, in general, considerably less than, for example, that of those employed in wood preservation in Hawaii [2].

ADDENDUM

While this manuscript was in preparation, two related methods were published. A reversed-phase LC method was described for confirmation of the presence of different chlorinated phenols in the urine, using electrochemical detection [22]. Di-, tri-, tetra- and pentachlorophenols could be separated with an LC run of approximately 1.5 h. In the other method, chlorophenols in tissue specimens were analyzed with high-performance LC on silica [23].

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