The Electron-Capture Gas Chromatography of Paradichlorobenzene Metabolites as a Measure of Exposure

by J. D. McKINNEY and L. FISHBEIN
National Institute of Environmental Health Sciences
Research Triangle Park, N. C.
and
by C. E. FLETCHER and W. F. BARTHEL
Atlanta Toxicology Branch, Food and Drug Administration
Atlanta, Georgia

Paradichlorobenzene has been used extensively as a household moth control agent and as a deodorant for many years, and it is considered to be safe and suitable for these purposes. However, cases of poisoning attributed to paradichlorobenzene have been reported (1-4). A convenient method for determining exposure to this halobenzene is important in evaluating any hazards involved in its use. The halobenzenes are metabolized mainly by oxidation to phenols, quinols, catechols and related compounds, or by forming mercapturic acids.

Various workers have reported (2,5) that the primary metabolites of paradichlorobenzene (PDB) are 2,5-dichlorophenol (DCP) and 2,5-dichloroquinol (DCQ). They are excreted in the urine as glucuronide and ethereal sulfate conjugates. PDB differs from its o- and m-isomers in that none of its metabolites are mercapturic acids (6). Furthermore, unlike PDB, the o- and m-isomers have metabolites that are qualitatively similar to those of the monochlorobenzenes although they differ completely quantitatively (7).

The high volatility and reactivity of PDB complicates its detection; however, the determination of its less volatile and reactive metabolites, DCP and DCQ, would not be as difficult. Existing methods (8-13) for the determination of halogenated phenols, quinols, etc., including some GLC (gas liquid chromatography) methods, are less sensitive and dependable than that desired. This paper describes a method which successfully employs gas chromatography with electron capture detection for the measurement of PDB and its major metabolites.

Experimental Procedure

Animal Dosing

New Zealand white rabbits were given a single dose of 0.5 g/kg of PDB [dosage identical to that used by Azouz et al. (5)] by stomach tube; blood samples were obtained 1 day, 2 days, and 3 days following the single dose and urine samples were collected in the same manner as that of Azouz et al. (5). Blood and urine samples were also obtained from control rabbits. All blood samples were immediately centrifuged and refrigerated prior to analysis.

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Methods

A 5-ml urine sample was acidified with 0.5-ml of concentrated hydrochloric acid (HCl). This solution was shaken at room temperature for 30 minutes. The acidic solution was then extracted with two 5-ml portions of pesticide quality benzene. In some samples it was necessary to add NaCl or $(NH_4)_2SO_4$ to prevent or reduce emulsion formation. The combined benzene extracts were dried over anhydrous sodium sulfate (Na_2SO_4). The necessary concentrations for GLC analysis were made by diluting portions of this dried solution.

A 5-ml whole blood sample was treated in the following manner. The sample was centrifuged at 10°C and 600 x g to separate the red blood cells from the plasma. The plasma layer (2.0 to 2.5 ml) was extracted with two 3-ml portions of pesticide quality benzene. The combined extracts were then dried over anhydrous Na_2SO_4 and subjected to GLC analysis without further dilution.

GLC Columns

The GLC analyses were made on a Varian Aerograph model 2100 gas chromatograph equipped with a tritium detector. Two columns were required for the complete analysis, and these were used interchangeably with the tritium detector and variations in column temperature for the desired analysis. PDB and DCP were analyzed directly using 15% FFAP (half ester of 2-nitroterephthalic acid-carbowax) as the stationary liquid phase with 40 to 50 mesh Chromasorb W as the supporting phase. The column material was prepared by dissolving 15 grams of FFAP in 250 to 300 ml of methylene chloride. The solution was transferred to a round bottom flask to which was added 85 grams of 40 to 50 mesh Chromasorb W. The slurry was freed of methylene chloride by rotary evaporation and the resulting solid material was dried overnight in an oven at 150°C. A 6' x 1/8" silanized glass column was then packed with this material.

DCQ was analyzed directly using 3% DEGS (diethylene glycol succinate) + 1% phosphoric acid on 30 to 60 mesh Chromasorb W. This column material was prepared by dissolving 3 grams of DEGS in 50 to 100 ml of acetone to which was added a solution of 1 gram of phosphoric acid in 50 ml of acetone. Ninety-six grams of 30 to 60 mesh Chromasorb W was added to the resulting solution. The slurry was freed of acetone by rotary evaporation, dried and packed in a 3' x 1/8" silanized glass column.

GLC Analysis

The gas chromatographic parameters necessary for each analysis are listed in Table I. Both columns were baked for 16 hrs. at 200°C prior to conditioning. Nitrogen was used as the carrier gas throughout the analysis.

Standard solutions (3 to 5 nanograms per microliter) of PDB, DCP and DCQ were prepared in suitable pesticide quality solvents (used throughout this work) and subjected to GLC analysis.

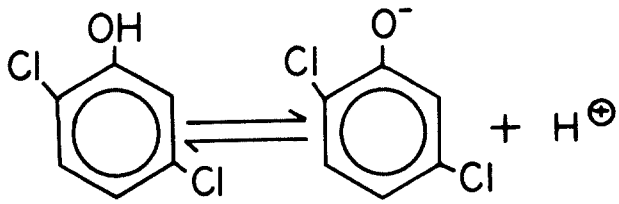
TABLE I

Gas Chromatographic Conditions

Compound	Column Material	Oven Temp. °C	Detector Temp. °C	Inlet Temp. °C	Flow Rate cc/min.
PDB	15% FFAP	140	200	225	45
DCP	15% FFAP	170	200	225	45
DCQ	3% DEGS + 1% H_3PO_4	170	200	225	45

Results and Discussion

Low volatility and high adsorption properties of phenolic pesticides has limited their direct analysis on silicone columns. It was observed in our laboratory that the phenolic and quinolic metabolites of PDB could be analyzed directly using polar liquid phases which permitted low adsorptivity (14,15). The ability of these materials to furnish protons or positive sites, thereby shifting the thermally influenced chemical equilibrium of the substance in the direction of its unionized (associated) form, permitted the direct analysis.



In the case of DCQ this was accomplished with good sensitivity while still retaining symmetrical peaks using a DEGS column with added phosphoric acid. In the case of DCP a higher degree of adsorptivity was achieved using FFAP. The availability of protons (positive sites) in FFAP is due to the electron withdrawing effect of the nitro group on the "free" carboxyl group. Table II presents the retention times and sensitivity of the method. The urine analyses gave 186 ppm of DCP and 2.6 ppm of DCQ with no detectable PDB. The numbers given are the average values obtained from two to four determinations. Typical chromatograms for standards, control urine and experimental urine are shown in Figures I-III. The overshoot shown on the chromatograms for the FFAP column diminished with repeated injections and did not significantly reduce the standing current of the gas chromatograph.

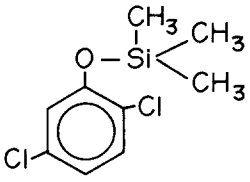
TABLE II

Gas chromatography data for PDB, DCP, and DCQ (using conditions shown in Table I).

Compound	Retention Time (min)	Nanograms Injected	% Deflection of Full Scale	Method Sensitivity (peak height)
PDB	1.2	5	65	0.38 ng/cm
DCP	7.3	5	45	0.55 ng/cm
DCQ	7.0	0.5	45	0.06 ng/cm

The presence of DCP in the urine was qualitatively confirmed by pretreatment of the sample to form the silyl ether derivative (I). The derivative was determined on a 3' aluminum column of 5% DC-200 on 60 to 80 Chromasorb G at 138° installed in a Varian Aerograph 1525 B gas chromatograph. Although quantification by this method was poor, this work provided information pertinent to the work-up procedure following silylation (16) of phenolic residues. For example, an aqueous extraction of the hexane silylation reaction solution affords a much cleaner chromatogram and does not significantly lower the concentration of the desired silyl ether through hydrolysis; however, hydrolysis did yield a siloxane (II) peak which could be used as an internal standard from which relative retention times could be calculated (I, RRT = 1.6 min; III, RRT = 6.8 min).

This silylation method was not successful in confirming the presence of DCQ in rabbit urine probably due to difficulty in silylating both hydroxyl groups quantitatively as well as the low concentrations of this metabolite present. However, standard solutions of DCQ were converted into a silyl derivative which was then chromatographed on the 5% DC-200 column. A relatively long retention time obtained for this derivative suggested that it might have been the monosilyl derivative (III).



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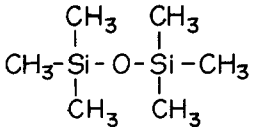

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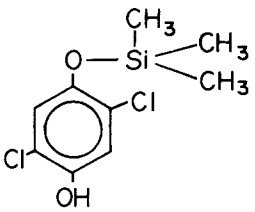

III

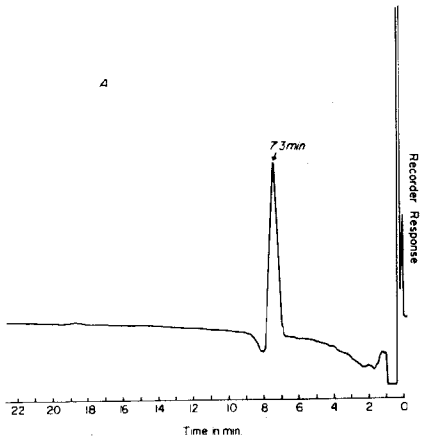

Figure I Standard Solutions of DCP and DCQ
A) 5 ng of DCP on 15% FFAP
B) 0.5 ng of DCQ on 3% DEGS/
1% H_3PO_4

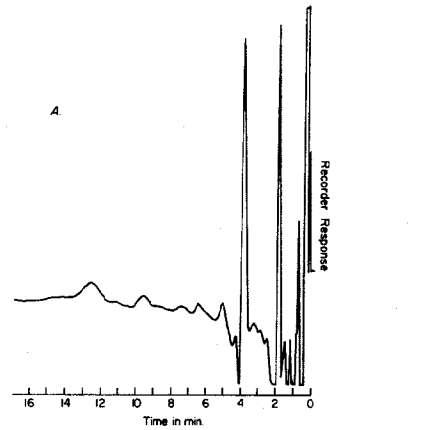

Figure II Control Urine
A) 1 microliter on 15% FFAP
B) 5 microliters on 3% DEGS/
1% H_3PO_4

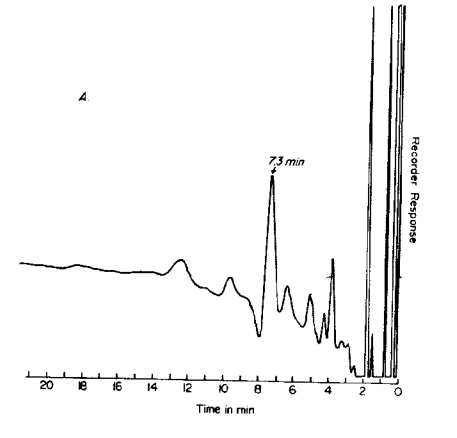

Figure III. Experimental Urine
A) 1 microliter on 15% FFAP
B) 5 microliters on 3% DEGS/
1% H_3PO_4

Previous workers (5) who were primarily interested in qualitative identification of PDB metabolites had used vigorous hydrolysis with refluxing concentrated hydrochloric acid (HCl) and steam distillation in order to free DCP and DCQ from its conjugates. These conditions would not only favor loss of the materials due to volatilization but would also facilitate further reaction and/or decomposition. Betts et al. (17) have shown that pentachlorophenol levels in rabbit urine are not significantly increased by hydrolysis with 5N-HCl before steam distillation beyond those levels obtained by steam distillation of the urine adjusted to pH 1; therefore, if conjugates form at all, they must be very labile to acid. In analogy with pentachlorophenol, the conjugates of DCP and DCQ might also be labile. Furthermore, Ishidate et al. (18) have shown that the electronically analogous N-glucuronide derived from aniline can be hydrolyzed on a column simply by eluting with 1N HCl. Spiked urine samples were subjected to a variety of extraction solvents and workup procedures, including those used by Azouz et al. (5), to obtain conditions affording the highest recovery percentages. However, none of the variations attempted gave a significant increase in the recovery percentages obtained by the simple and convenient procedure described in this paper.

TABLE III

% Recoveries from Spiked Samples

	PDB	DCP	DCQ
a) Urine	78	92	85
b) Serum	94	87	0

As shown in Table III this extraction procedure failed to recover DCQ from spiked serum samples. Other extraction procedures as well were unsuccessful in recovering DCQ from serum suggesting that it may be tightly bound to blood components. This can be attributed to the apparent lability of DCQ to autooxidation as evidenced by increasing coloration of its standard solutions with time. The p-quinoidal system formed would greatly enhance the binding propensities. PDB nor its metabolites were found in any of the blood werum samples from dosed rabbits.

Although our control samples indicated that it was not necessary in our analysis to include a basic extraction followed by acidification and re-extraction as is normally done in the cleanup procedure for isolating phenolic metabolites, further cleanup may be desirable to increase the lifetime of the columns if these analyses are performed routinely. However, a significant loss of sensitivity did not occur with these columns after several months

of repeated injections under the conditions described in Table I.

No attempt was made to establish the percentage of the dose represented by the various levels of metabolites found, nor was any attempt made to determine the levels of glucuronide and ethereal sulfate conjugates before hydrolysis. Previous workers (5) have shown that PDB nor its metabolites are excreted in feces. In considering a practical application of the method, it would be difficult to determine the exact dosage an individual received from exposure; therefore, it would be difficult to ascertain the percentage of the dose excreted as metabolites. The method will hopefully permit determination of "tolerable" levels of exposure as measured by excreted metabolites prior to acquiring toxic symptoms. This more sensitive and convenient method for determining exposure to PDB via measurement of the major urinary metabolites may also be advantageous in elucidating the mode and mechanism of action of PDB, and other halobenzenes, and may have general application for the direct GLC determination of chlorinated phenols with electron capture detection.

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