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Note

Identification of polychlorinated phenols in urine by gas and thin-layer chromatography

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A sensitive analytical screening and identification procedure for chlorinated phenols in biological material is of great importance in clinical toxicology, occupational medicine and environmental control. These phenols can be a direct source of intoxication or can originate in vivo by oxidative biodegradation of chlorinated chemicals used as pesticides [1–8].

The volatility of chlorinated phenols is not sufficient for sensitive analysis by gas chromatography using the headspace technique and an extractive method is more convenient. The biotransformation of these phenols involves conjugation, so a hydrolysis step should precede the extraction [8–11]. To minimize isolation losses of these phenolics, which are partly volatile weak acids, alkaline hydrolysis seems to be more reliable than acid hydrolysis [12].

A method of isolation and identification was developed in experiments with rats administered a single 67 mg/kg oral dose of lindane (γ-hexachlorocyclohexane). Gas chromatography (GC) with electron-capture detection (ECD) has been used previously to evaluate the degree of conjugation and the dynamics of the excretion of the main phenolic metabolites into urine [12,13].

The separation of all isomers of chlorinated phenols as acetyl derivatives by gas chromatography has been described [14], but has not been widely applied to biological material [1–6, 8]. This paper describes a simple combination of gas and thin-layer chromatography (TLC) for the identification of polychlorinated phenols in urine. Acetyl derivatives of phenols for gas chromatography were prepared by the described procedure [12, 15, 16].

EXPERIMENTAL

Chemicals

Analytical-reagent grade chemicals were used unless indicated otherwise.

n-Hexane was obtained from Park (Northampton, U.K.) and methanol, benzene, acetone, acetic acid anhydride, potassium carbonate, potassium hydroxide, hydrochloric acid, 26% ammonia solution and silver nitrate from Lachema (Brno, Czechoslovakia). Light petroleum (b.p. 30–60 °C) was purchased from Reactivul (Bucharest, Romania).

Lindane (technical grade) was obtained from Spolana (Neratovice, Czechoslovakia). Standards of chlorinated phenols were partly a generous gift from Ciba-Geigy (Basle, Switzerland) and partly from Dr. Gajdůšková, Institute of Veterinary Medicine, Brno, Czechoslovakia.

Chromatographic materials

Silufol plates were obtained from Kavalier (Votice, Czechoslovakia) and 3% SP 2250 on Supelcoport (80–100 mesh) from Supelco (Gland, Switzerland).

Isolation of phenols and derivatization for GC with ECD

A mixture of 0.5 ml of urine and 0.5 ml of 5 *M* potassium hydroxide solution was boiled under reflux for 20 min. After cooling, 0.4 ml of hydrolysate were separated, acidified with 0.2 ml of 6 *M* hydrochloric acid and then extracted with 3 ml of *n*-hexane. In the cases of heavy experimental intoxication examined here, it was sufficient to analyse, using sensitive ECD, only one sixth of the *n*-hexane extract without further concentration, but different amounts could be used according to the actual need. For the acetylation reaction, therefore, only 0.5 ml of the *n*-hexane extract was made alkaline with 0.5 ml of 0.1 *M* potassium carbonate solution (pH 10.5), prepared daily and shaken with 10 μl of acetic anhydride for 10 min [12]. The layers were separated by centrifugation and 1 μl of the *n*-hexane layer was injected into the gas chromatograph.

The recovery of the whole isolation procedure, including the hydrolysis step, was calculated on the basis of urine spiked with 2,3-dichlorophenol (DCP), 2,4,6-trichlorophenol (TCP) and 2,3,5,6-tetrachlorophenol (TeCP) to be 95% on average.

Isolation of phenols for TLC

A 7-ml volume of urine from intoxicated rats and 7 ml of 5 *M* potassium hydroxide solution were boiled under reflux for 20 min. After cooling, 10 ml of hydrolysate were separated, acidified with 5 ml of 6 *M* hydrochloric acid and extracted with 30 ml of *n*-hexane. The *n*-hexane layer was separated and evaporated to a volume of about 0.5 ml by heating at 80 °C. The final phenol extract was therefore ten times more concentrated that the original urine. In cases of mild human intoxication a greater amount of urine should be taken for isolation (20 ml).

GC and TLC procedure

Extracts of chlorinated phenols were analysed simultaneously by TLC on Silufol plates and by GC as acetyl derivatives on a glass column packed with 3% SP 2250 on Supelcoport (80–100 mesh) operated isothermally at 160 °C with ECD as described [12].

For identification of individual phenolic metabolites by TLC, the following mobile phases were used: (1) benzene–methanol (95:5, v/v); (2) light petroleum–benzene (1:1, v/v); (3) *n*-hexane–acetone (30:10, v/v); (4) benzene–chloroform (70:30, v/v); and (5) *n*-hexane–benzene–ethyl acetate (6:4:1, v/v).

Sensitive detection was achieved using an ammoniacal acetone solution of silver nitrate [17] followed by exposure to UV light for 5 min.

RESULTS AND DISCUSSION

Fifty urine samples from ten pairs of rats intoxicated orally with a single dose of lindane were analysed. In all urine portions analysed by GC and TLC the contents of phenolic metabolites decreased in the order 2,3,5,6-TeCP, 2,4,6-TCP, 2,4,5-TCP and 2,3-DCP.

We obtained retention data for DCP, TCP, TeCP (except 2,3,4,6-TeCP) and pentachlorophenol (PCP) in order to permit the reliable identification of the main phenolic metabolites of lindane and possibly other chlorinated pesticides.

These data for the acetyl derivatives obtained on a packed column of medium polarity are summarized in Table I. TLC R_F values of phenols in three mobile phases selected as examples are presented in Table II. An example of the GC separation of acetylated standards of chlorophenols is shown in Fig. 1 and of those isolated from rat urine in Fig. 2.

Some isomers of DCP, TCP and TeCP are not separated by GC on a packed column of medium polarity (Table I), nor are they separated on polar or nonpolar capillary columns [14]. It is clear that an unambiguous identification of these

TABLE I

GC RETENTION DATA OF ACETYL DERIVATIVES OF CHLORINATED PHENOLS

Glass column (1.25 m × 3 mm I.D.) packed with 3% SP 2250 on Supelcoport (80–100 mesh) operated isothermally at 160 °C; carrier gas (nitrogen) flow-rate, 35 ml/min. Absolute retention time of 2,4,6-trichlorophenyl acetate, 2.25 min.

Acetate	Relative retention time	Acetate	Relative retention time
3,5-DCP	0.63	2,4,5-TCP	1.30
2,4-DCP	0.67	2,3,6-TCP	1.32
2,5-DCP	0.67	3,4,5-TCP	1.67
2,6-DCP	0.67	2,3,4-TCP	1.80
2,3-DCP	0.81	2,3,5,6-TeCP	2.37
3,4-DCP	0.83	2,3,4,5-TeCP	3.04
2,4,6-TCP	1.00	PCP	5.26
2,3,5-TCP	1.28		

TABLE II

TLC R_F VALUES OF CHLORINATED PHENOLS ON SILUFOL

Mobile phases: I, benzene–methanol (95:5, v/v); II, light petroleum–benzene (1:1, v/v); III, *n*-hexane–acetone (3:1, v/v).

Chlorophenol	R_F value		
	I	II	III
2,3-DCP	0.51	0.26	0.29
2,4-DCP	0.49	0.24	0.33
2,5-DCP	0.52	0.32	0.38
2,6-DCP	0.70	0.43	0.45
3,4-DCP	0.32	0.10	0.37
3,5-DCP	0.40	0.12	0.43
2,3,4-TCP	0.45	0.21	0.38
2,3,5-TCP	0.51	0.27	0.40
2,3,6-TCP	0.72	0.37	0.47
2,4,5-TCP	0.49	0.22	0.44
2,4,6-TCP	0.68	0.40	0.52
3,4,5-TCP	0.38	0.10	0.44
2,3,4,5-TeCP	0.49	0.19	0.51
2,3,5,6-TeCP	0.72	0.41	0.43
PCP	0.69	0.34	0.50

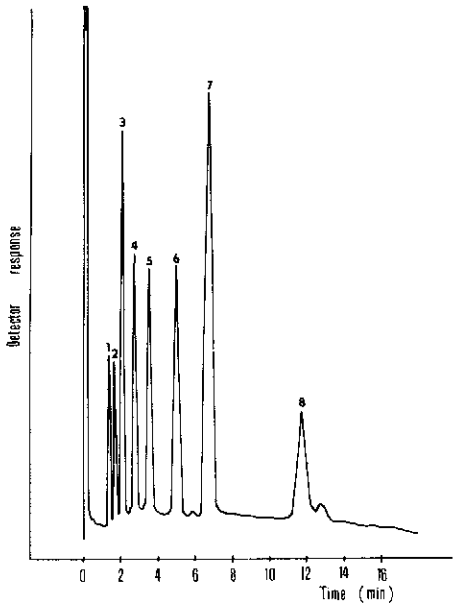

Fig. 1. Gas chromatogram of 1 μl of acetylated standard mixture containing 400 pg each of (1) 2,6-DCP, (2) 2,3-DCP, (3) 2,4,6-TCP, (4) 2,4,5-TCP, (5) 3,4,5-TCP and (6) 2,3,5,6-TeCP, 800 pg of (7) 2,3,4,5-TeCP and 200 pg of (8) PCP. Attenuation, 16; recorder range, 50 mV.

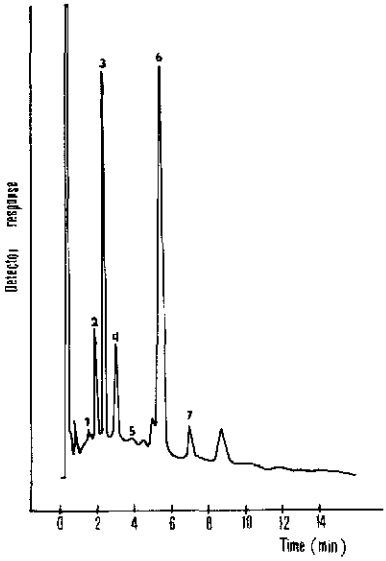

Fig. 2. Gas chromatogram of 1 μl of extract of hydrolysed rat urine 72 h after lindane intoxication. Peaks as in Fig. 1. Attenuation, 16; recorder range, 50 mV.

metabolites on the basis of GC alone would not be possible. TLC confirmed the presence of 2,3-DCP and 2,4,5-TCP in all the samples of rat urine by excluding the other possibilities. Some uncertainty remains regarding the identity and purity of 2,3,5,6-TeCP, which need not be separated from 2,3,4,6-TeCP by GC [14]. However, in agreement with retention data from liquid chromatography on silica [18], the possibility of the separation of the two isomers by TLC is apparent. Nevertheless, as 2,3,4,6-TeCP was not available as a reference standard, its presence cannot be completely excluded. Both isomers are mentioned in the literature as possible metabolites of lindane [6,7].

The sensitivity of GC with ECD was 10^{-3} ppm [12] and that of TLC was 1 ppm. The sensitivity of the latter method is comparable to that of GC with flame ionization detection. If necessary it would be possible to increase the sensitivity of TLC by extraction of a larger volume of urine.

The sensitivity of the TLC method would be adequate for application in a laboratory of clinical and forensic toxicology to cases of suspected intoxication by chlorinated phenols or their precursores, chlorinated pesticides, as can be seen from a case report (Fig. 3). A 2-year-old child was suspected to have ingested a pesticide containing *p*-dichlorobenzene. A 20-ml volume of urine was submitted to alkaline hydrolysis and the hydrolysate was investigated for chlorophenols by the described procedure. The spot of the suspected metabolite 2,5-DCP after TLC separation was cut from the Silufol plate, extracted with 1 ml of *n*-hexane, acetylated and 1 μl was injected into the gas chromatograph. The presence of this metabolite in the urine was confirmed.

The combination of GC and TLC can be a helpful tool for the identification of unknown substances in a toxicological laboratory.

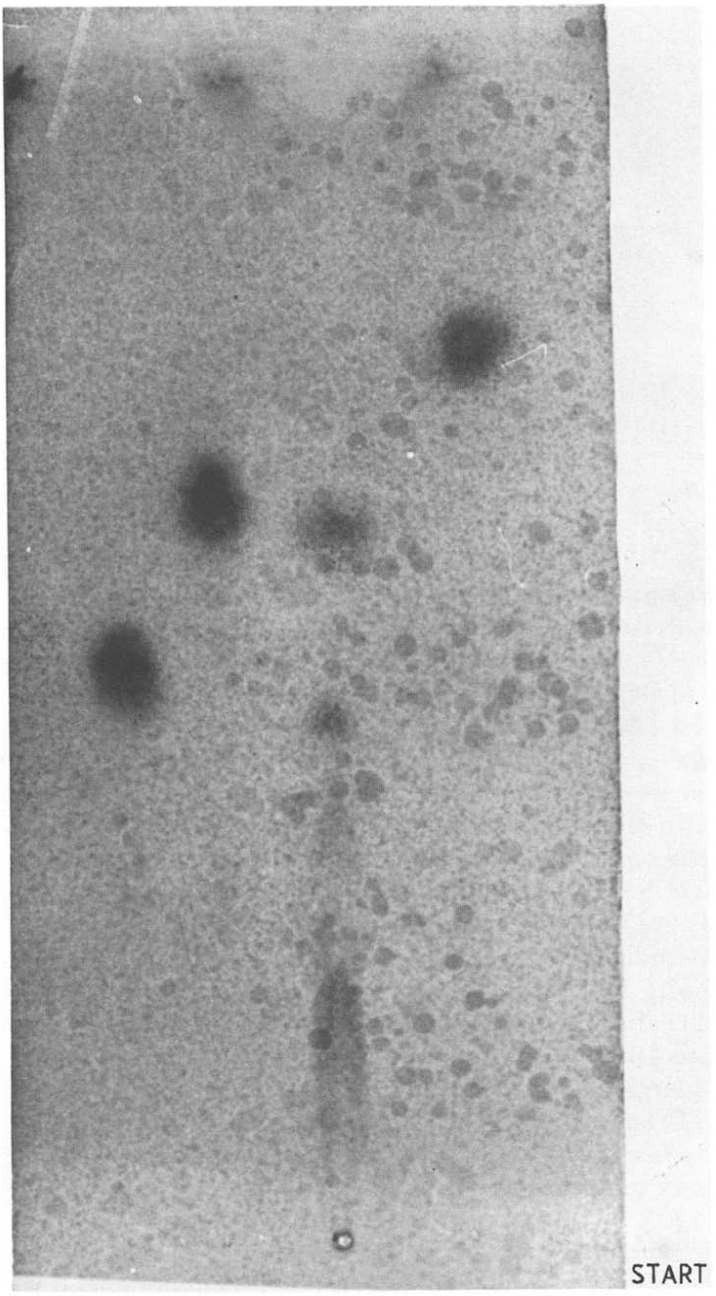

Fig. 3. Results for the case of a child suspected of ingestion of *p*-dichlorobenzene: confirmation of the metabolite 2,5-DCP in urine. TLC of urine extract after alkaline hydrolysis. Adsorbent, Silufol; mobile phase, *n*-hexane–benzene–ethyl acetate (6:4:1, v/v); detection reagent, silver nitrate in acetone, UV illumination. Samples, from left to right: 2,4-DCP, 2,5-DCP, extract of hydrolysed urine, 2,6-DCP.

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