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Note

Improved assay for the rapid determination of bis(*p*-chlorophenyl)acetic acid in human urine

H. DELLA FIORENTINA

Laboratoire Provincial-Toxicologie du Travail, 151, Bd. de la Constitution, 4020 Liège (Belgium)

J. DE GRAEVE

Laboratoire de Chimie Médicale, Université de Liège, Unité de Biochimie, Batiment B 23, Institut de Pathologie, B-4000 Sart-Tilman par Liège 1 (Belgium)

and

M. GROGNA and F. DE WIEST

Laboratoire de Toxicologie de l'Environnement, Université de Liège, 151, Bd. de la Constitution, 4020 Liège (Belgium)

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For the past decade, the potential health hazard of persistent chlorinated hydrocarbon insecticides to human beings has been a subject of primary interest[1]. The trichlorobis(*p*-chlorophenyl)ethane family of compounds, certainly the most widely used insecticides since World War II, constitute a serious problem, not only for workers exposed to them in factories but also for the general population.

The analysis of residues of these chlorinated insecticides and the quantitative measurement of their main metabolites have stimulated the development of sensitive and accurate analytical methods. According to many authors[2–4], the urinary excretion of bis(*p*-chlorophenyl)acetic acid (DDA), a relatively water-soluble metabolite, could be used as an indicator of exposure to DDT. In comparison to the conventional methods for measuring DDT and other metabolites in fatty tissues or blood samples, the great advantage of systematic monitoring of DDA in human urine becomes obvious.

In previous papers, DDA excretion levels were estimated either by a tedious spectrophotometric procedure after ion-exchange purification[5] or by a gas–liquid chromatographic (GLC) separation after methylation of the carboxylic function with 10% boron trifluoride in nanograde methanol[6]. The latter method could be applied with the Coulson conductivity detector or with an electron-capture detector (ECD). This procedure requires a time-consuming microflorisil clean-up step[7].

This paper reports modifications of the GLC method which improve its rapidity, accuracy and selectivity for the detection of DDA in human urine.

EXPERIMENTAL

Chemicals

All the chemicals and solvents were of analytical grade supplied by Carlo-Erba

(Milan, Italy) and were used without further purification. Florisil for column chromatography was purchased from E. Merck (Darmstadt, G.F.R.), DDA and Diazald 99% from Aldrich-Europe (Beerse, Belgium). Diazomethane in diethyl ether was prepared monthly following the usual technique[8]. Boron trifluoride in propanol (14%, w/v) was obtained from Applied Science Labs. (Mornard, Belgium). The different esterification procedures were performed according to the suppliers' technical notes[9,10].

Apparatus

All the glassware was cleaned in acids and rinsed with distilled water and solvents prior to a final check for sample contamination.

A Tracor Model 550 gas chromatograph equipped with a 10-mCi ^{63}Ni pulse-frequency-modulated ECD was used. The pulse mode was selected at 55 V (pulse width, 3 μsec; pulse rate, 270 μsec). The instrument was operated with a glass column (Pyrex 6 ft. $\times$ 0.25 in. O.D.) packed with 1% QF-1 on Gas-Chrom Q (100–120 mesh). The flow-rates of both the carrier and make-up gas (argon–methane, 90:10) were regulated at 30 ml/min. The temperatures of the injector block, column oven and detector were 250°, 190° and 290° respectively. Gas chromatographic–mass spectrometric (GC–MS) analysis was performed on a LKB 9000 S instrument. The chromatographic separation conditions were identical to those previously described. The temperatures of the separator and ion source were 260° and 270°, respectively. The mass spectra were recorded with 70-eV incident electrons and a 60-μA trap current.

Procedure

Urine samples (5–50 ml depending on the degree of DDT exposure) were mechanically extracted for 10 min in test-tubes or funnels with an equal volume of a 2% acetic acid solution in hexane. After centrifugation at 1155 *g* for 5 min, the upper organic phase was transferred to a conical tube. The remaining aqueous phase was re-extracted with the same quantity of solvent mixture.

The combined organic phases were carefully dried over anhydrous sodium sulphate and evaporated to dryness *in vacuo* at 50° to eliminate the remaining traces of water. Methylation of the residue was performed at room temperature with 500 μl of an ethereal solution of diazomethane. After 15 min, the resulting solution was evaporated to dryness under a nitrogen stream. The residue was finally dissolved in an appropriate volume of hexane containing 0.1 μg/ml DDA propyl ester as internal standard. An aliquot (1–5 μl) was injected into the gas chromatograph and the integrations of both the DDA methyl and propyl ester peaks were used for the quantitative measurement. Standard calibration curves were prepared by adding known amounts of DDA to human urine free from DDA (Fig. 1).

RESULTS AND DISCUSSION

Several derivatization and isolation techniques were applied to the same spiked urine specimens in order to eventually bypass the tedious and time-consuming clean-up procedure which is recommended for the DDA determination by electron-capture gas chromatography[7].

Figs. 2 and 3 show different chromatograms recorded under optimal conditions.

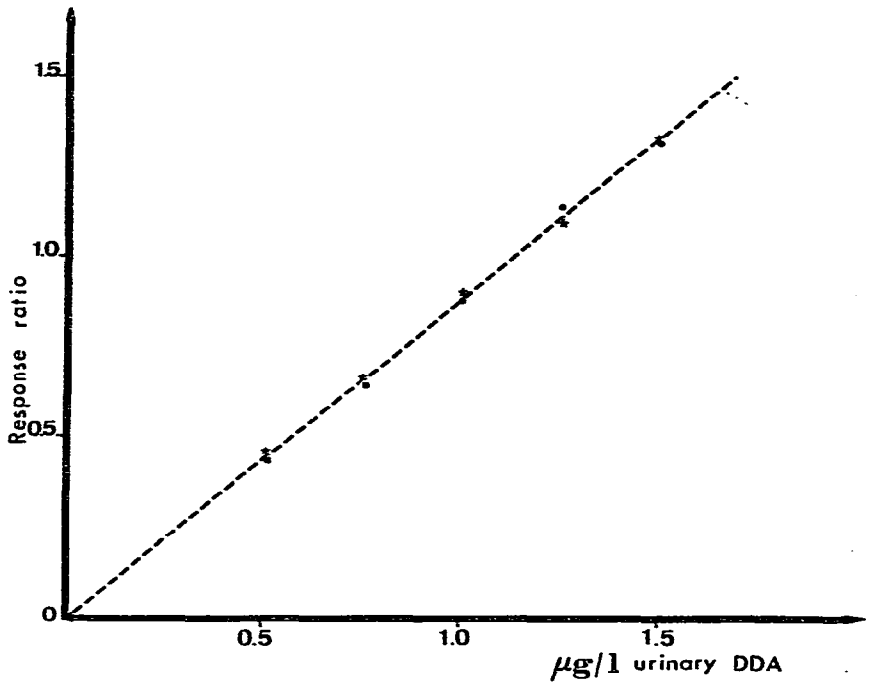

Fig. 1. Standard calibration curve for DDA determination in urine (★) and water (●) samples using 0.1 μg/ml of DDA propyl ester as internal standard. Attenuation: $4 \cdot 10^{-10}$ A. Full scale 1 mV recorder.

The former was obtained on a sample derivatized with boron trifluoride–methanol (A = crude extract; B = after a microflorisil clean-up procedure), the latter after diazomethane derivatization. From these figures, the following observations can be made. (1) After treatment with boron trifluoride–methanol, it is clear that the microflorisil column purification is required in order to avoid the elution of many interfering peaks which result in a low efficiency of chromatographic separation. In addition, the presence of several compounds having low volatility greatly increases the delay between analyses. (2) In contrast, the diazomethane esterification leads to cleaner chromatographic traces where eventual interferences from endogenous or exogenous compounds are significantly reduced.

In conclusion, the diazomethane methylation technique constitutes an important improvement of the methodology which, in practice, obviates the need for the clean-up step. Consequently, as described, our procedure is not only simpler but also enables the achievement of clear chromatographic traces thus reducing the risk of ECD contamination.

Precision. The variation coefficients of 10 urine samples containing 10, 50 and 100 μg/l were 10, 8 and 6%, respectively.

Accuracy. The accuracy of the assay was examined on spiked urines. From the data presented in Table I, a 0.998 correlation coefficient was obtained demonstrating the high accuracy of the method.

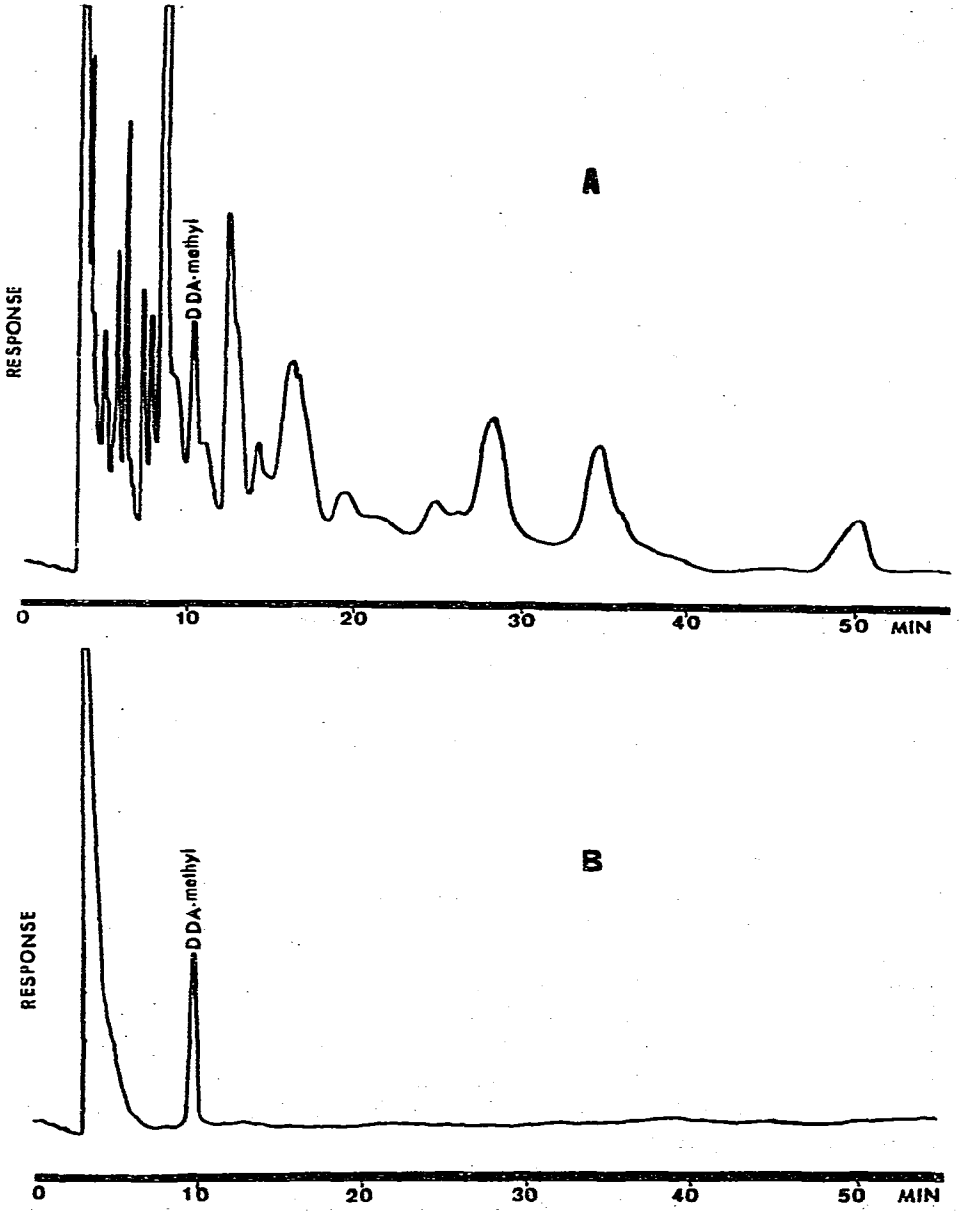

Fig. 2. GC separation of urinary extract of DDA methylated with boron trifluoride–methanol before (A) and after (B) microflorisil clean-up. For GLC conditions, see Experimental, except that the oven temperature was 160°.

Specificity. The specificity of our technique was assessed by GC–MS analysis of different urinary extracts obtained from people exposed to insecticides. Fig. 4 shows the mass spectrum recorded at the top of the GC peak corresponding to DDA methyl ester. It is absolutely identical to the spectrum obtained from the reference compound.

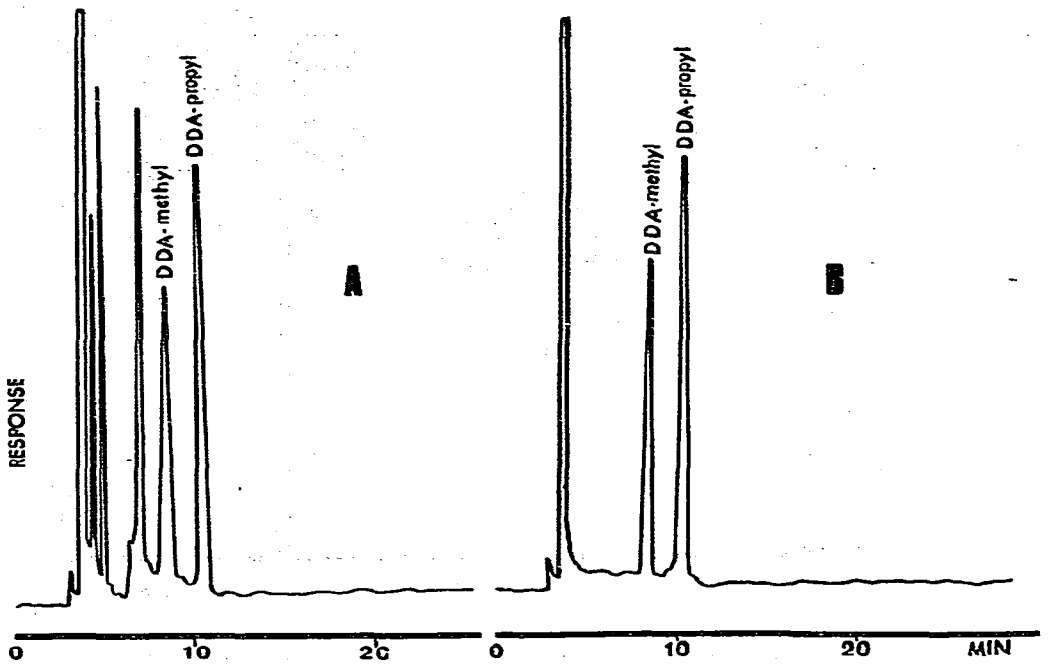

Fig. 3. Gas chromatograms of a urinary extract of DDA methylated with diazomethane before (A) and after (B) microflorisil clean-up. For GLC conditions, see Experimental.

TABLE I

ACCURACY OF THE DETERMINATION OF DDA

Sample	*Amount of DDA (μg/l)*	
	Added	*Found*
A	20	19
B	10	8
C	50	51
D	100	95
E	15	17
F	25	24
G	35	37
H	5	6

Rapidity. Since only a single extraction procedure is required in order to obtain a clear chromatographic trace with short retention times for DDA esters, a great number of samples (about 20–30) can be analyzed by one operator within a normal working day.

Sensitivity. The extraction yields of *ca.* 95% make this method very sensitive and totally suitable for the routine examination of human urine samples. The absolute detection limit is *ca.* 0.05 ng.

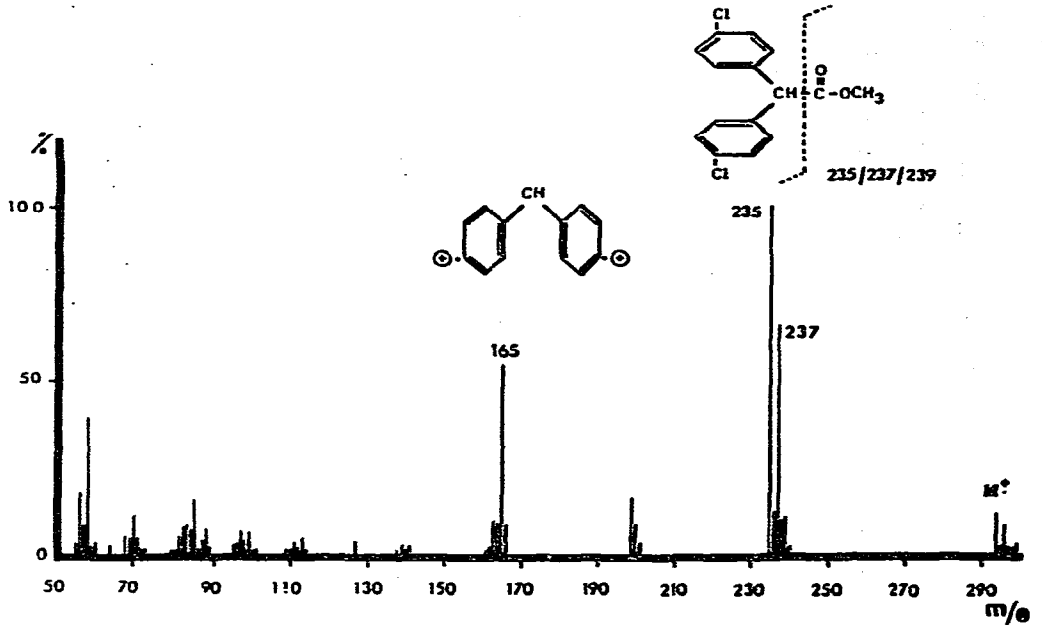

Fig. 4. Mass spectrum recorded at the top of the GLC peak corresponding to the DDA methylation.

Under our experimental conditions, the use of a suitable diazomethane methylation procedure in addition to an appropriate internal standard has yielded a simple, specific, rapid and precise technique for DDA determination in human urine. The main advantage of this method is the rapidity which allows at least 10 times as many analyses to be performed in comparison with the previous method of Crammer *et al.*[6].

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