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HIGH-PERFORMANCE LIQUID AND GAS CHROMATOGRAPHY OF DIALKYLPHOSPHATES, DIALKYLTHIOPHOSPHATES AND DIALKYLDITHIOPHOSPHATES AS THEIR PENTAFLUOROBENZYL DERIVATIVES

V. BARDAROV
Institute of Hygiene and Occupational Health, 1431 Sofia (Bulgaria)
and
M. MITEWA*
Department of Chemistry, University of Sofia, 1126 Sofia (Bulgaria)
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SUMMARY

High-performance liquid and gas chromatographic (GC) procedures for the separation and determination of dialkylphosphates, dialkylthiophosphates and dialkyldithiophosphates (alkyl = methyl or ethyl), the main hydrolytic metabolites of organophosphate pesticides, are described. The application of different procedures and their limits of detection are discussed. The GC proposed procedures were applied to the analysis of urine samples from humans exposed and not exposed to organophosphates.

INTRODUCTION

Dialkylphosphates (DAP), dialkylthiophosphates (DATP) and dialkyldithiophosphates (DADTP) (Fig. 1), being hydrolytic metabolites of organophosphorus (OP) pesticides, are excreted in urine[1–3]. Their level of excretion is a convenient indicator of human exposure to OP pesticides[3,4]. The application of such an indicator, however, requires the development of a suitable method for the routine simultaneous determination of all DAP, DATP and DADTP and in some instances also alkylphosphates, alkylthiophosphates, alkyldithiophosphates, thiophosphoric acid and dithiophosphoric acid.

A number of procedures have been developed, based on different derivatization procedures followed by gas chromatographic (GC) determination of the derivatives using a phosphorus-specific detector. The procedures involving alkylation with diazomethane[3,5], diazoethane[3] or diazopentane[6–8] proved to be inconvenient for routine analysis, however, owing to the high toxicity and thermal instability of the chemicals used, the strong interference from the inorganic phosphates present in the urine and the formation of more than one derivative of DATP. Alternative procedures, based on the methylation with phenyltrimethylammonium hydroxide in methanol[9,10], are applicable only to thio- and dithiophosphates. The third possibility, involving the

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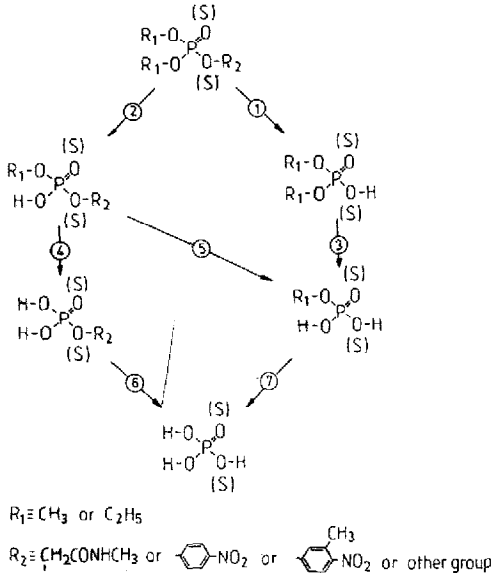

Fig. 1. Hydrolytic metabolism of organophosphates.

determination of the *p*-nitrobenzyl ester derivatives[11], excludes the use of nitrogen–phosphorus detection (NPD).

The determination of alkylphosphates following their conversion into their pentafluorobenzyl esters[12,13] seems to be the most convenient method, as these esters exhibit suitable GC properties and suffer no obvious interference from inorganic phosphates. In addition, the use of both NPD and flame-photometric detection (FPD) is possible. Nevertheless, some problems concerning the sample preparation, GC separation and the use of NPD hinder their application in routine analysis. The investigation of some of these problems and the application of high-performance liquid chromatography (HPLC) to dialkylphosphates are reported in this paper.

EXPERIMENTAL

Standards and reagents

Dimethyl phosphate (DMP), diethyl phosphate (DEP), the sodium salt of DMP and the potassium salts of dimethyl thiophosphate (DMTP), diethyl thiophosphate (DETP), dimethyl dithiophosphate (DMDTP) and diethyl dithiophosphate (DEDTP) were supplied by EPA (Research Triangle Park, NC, U.S.A.). Standard solutions were prepared weekly in acetonitrile [acetonitrile–water (99:1) for DAP and its sodium salt] and stored at 0–5°C.

2,3,4,5,6-Pentafluorobenzyl bromide (PFB-Br) (99+%) was obtained from Janssen (Nettetal, F.R.G.).

HPLC-grade pentane, methanol, ethanol and water and spectroscopic-grade cyclohexane and potassium bromide were used.

Acetonitrile, dichloromethane, acetone, heptane, anhydrous potassium carbonate, sodium perchlorate, sodium dihydrogenphosphate, dipotassium hydrogen-

phosphate and sodium hydroxide were of analytical-reagent grade. Tetraalkylammonium salts, tetraphenylarsonium chloride and dicyclohexyl-18-crown-6 were obtained from Fluka (Buchs, Switzerland) and Merck (Darmstadt, F.R.G.).

Liquid chromatography

Apparatus. The HPLC experiments were performed on a Perkin-Elmer LC system consisting of a series 4 pump, a 550 SE UV–VIS spectrometer with an 8-μl flow cell, a Rheodyne 7125 injection valve with a 300-μl loop for preparative work and a 20-μl loop for analytical work and an R-100 recorder. For electrochemical detection (ED) a Model 4B detector from Bioanalytical systems was used equipped with a glass carbon working electrode.

Chromatographic conditions. An anion-exchange column (250 × 4.6 mm I.D.) of SAX, 10 μm (Whatman, Clifton, NJ, U.S.A.) was used. The mobile phase was 0.05 *M* sodium perchlorate in 0.05 *M* phosphate buffer (pH 7.2) at a flow-rate of 1 ml/min and ED with a glassy carbon working electrode at a potential of 1.05 V *vs.* silver–silver chloride with 1 *M* potassium chloride reference electrode was used for the determination of DATP and DADTP.

The normal-phase (NP) preparative separation of PFB derivatives was performed with a 250 × 10 mm I.D. silica-50 (7 μm) column (Macherey, Nagel & Co., Düren, F.R.G.) with different mobile phases: 2% methanol and 2% ethanol in pentane for DAP-PFB separation, 1% methanol and 1% ethanol in pentane for DATP-PFB separation and 0.5% methanol and 0.5% ethanol in pentane for DADTP-PFB separation, with UV detection at 260 nm. In order to avoid fluctuation of the flow-rate an overpressure (helium, 30 p.s.i.) was applied in the mobile phase containers.

Gas chromatography

Apparatus. GC was performed on a Perkin-Elmer series 8300 instrument equipped with a heated injector for packed columns, a split–splitless injector for capillary columns, a nitrogen–phosphorus detector and a GP-100 printer.

Chromatographic conditions. The stationary phases used for packed columns were SE-30, DC-200, OV-17, OV-25, OV-225, QF-1, and Carbowax 20M and their binary mixtures coated on Chromosorb G HP (100–120 mesh) or Chromosorb W HP (100–120 mesh). The column temperature was held at 185°C for 8 min, then increased linearly to 240°C at 20°C/min. The carrier gas was nitrogen at a flow-rate of 35 ml/min. The temperature increase ensures both the elution of di- and tri(pentafluorobenzyl) phosphates and purification of the column when urine samples are analysed.

The capillary columns used were as follows: 25 m × 0.25 mm I.D. fused silica with Carbowax 20M, SP-1000 or OV-101 as the stationary phase and 50 m × 0.23 mm I.D. fused silica with polyvinylmethylsilicone (PVMS) as the stationary phase. The carrier gas was helium at a flow-rate of 3 ml/min. In all instances temperature programming was performed. In order to achieve high sensitivity, splitless injection was applied with the split flow valve in the "on" position 0.6 min after injection and with the column temperature programmed from 75 to 270°C (heptane was used as the solvent).

Detection. NPD in the phosphorus mode was applied at hydrogen flow-rate of 44 ml/min and, in order to achieve higher sensitivity, oxygen was used instead of air at a flow-rate of 32 ml/min when packed columns were used. NPD in the nitrogen–

phosphorus (NP) mode was applied with hydrogen and air flow-rates as recommended in the manual when capillary columns were used.

Additional apparatus

UV and IR spectra were recorded on Perkin-Elmer Lambda-5 UV–VIS and Perkin-Elmer 883 IR spectrometers (potassium bromide disks), respectively.

A rotary vacuum evaporator together with a thermostated water-bath, dry thermostat and Supelco Microware G/G kit (Part No. 6-4692) were used.

PFB derivatization of standards

Standard solutions of DAP, DATP and DADTP (treated in the same manner as the samples) and their PFB derivatives, methyldi(pentafluorobenzyl) phosphate [$MP(PFB)_2$] and tri(pentafluorobenzyl) phosphate [$P(PFB)_3$] solutions were used for calibration of the retention times and response factors.

Derivatization was performed in a reaction mixture consisting of DAP (or DATP or DADTP), PFB-Br and potassium carbonate (as catalyst) in acetonitrile or acetone after incubation at an appropriate temperature. The PFB derivatives were isolated by extraction with heptane. A typical preparative procedure was incubation of 1–5 mg of the appropriate phosphate, 40–60 μl of PFB-Br and 30–60 mg of potassium carbonate in 0.5 ml of acetonitrile (for DAP derivatization) or 1 ml of acetone (mainly for DATP and DADTP derivatization) for 0.5–3 h at 40–90°C, followed by extraction with heptane (wnen acetone was used as the solvent it was purged before extraction).

Sample preparation

(1) Extractive alkylation according to Bradway et al.[12]. Sealed stoppered tubes containing 1.0 ml of urine, 2 ml of dichloromethane, 1 ml of a 0.1 *M* aqueous solution of the ion-pair reagent and 30 μl of PFB-Br were shaken at ambient temperature for 1 h. After centrifugation, the organic phase was dried with 0.5 g of anhydrous sodium sulphate, 1 ml of it was evaporated under nitrogen to a residue of *ca.* 30 μl and then 0.50 μl of heptane and 5 μl of 10% sulphuric acid were added. Aliquots of the organic phase were analysed by GC.

(2) Procedure of Reid and Watts[13]. A 1.0-ml sample of urine and 7.0 ml of acetonitrile were mixed and centrifuged, 4.0 ml of the supernatant were transferred into a 10-ml reaction vessel and the solvent was removed using a rotary vacuum evaporator at 30–40°C or by means of a nitrogen flow at 60°C. The derivatization was performed by adding 0.5 ml of acetone and 20 μl of PFB-Br and incubation at ambient temperature for 30–60 min in a sealed reaction vessel. Then the acetone was removed with a nitrogen flow and the PFB derivatives of DATP and DADTP were extracted with 0.50 ml of heptane. The supernatant was separated after centrifugation and an aliquot (0.30 ml) was transferred into a sample vessel. The derivatization procedure was repeated for the residue (kept in the reaction vessel) at higher temperature: 0.5 ml of an acetonitrile solution of dicyclohexyl-18-crown-6 (0.2%), 30 mg of potassium carbonate and 20 μl of PFB-Br were added and the vessel was scaled and incubated for 1–2 h at 90°C. Then 0.50 ml of heptane and 5 ml of 10% sulphuric acid were added and 0.30 ml of the organic phase was taken and added to the first aliquot in the sample vessel.

RESULTS AND DISCUSSION

The absorption spectra and molar absorptivities of DMP, DMTP and DMDTP are shown in Fig. 2A and B. The molar absorptivities are moderate for DMTP and DMDTP and negligible for DMP in the non-specific region of the UV spectrum (200–220 nm), thus limiting significantly both the sensitivity and selectivity of their HPLC determination with spectrophotometric detection. On the other hand, the electrochemical activity of DMTP, DETP, DMDTP and DEDTP (Fig. 2C) makes HPLC determination with ED possible. The separation achieved for DMTP, DETP, DMDTP and DEDTP by means of ion-exchange HPLC with ED at a glass carbon working electrode potential of 1.05 V is demonstrated in Fig. 2D. It is evident that at potentials higher than 1.15 V for DMTP and DETP and higher than 1.00 V for DMDTP and DEDTP their direct determination is possible at concentrations less than 2 nmol/l. The existence of many other electrochemically active components (at the potentials mentioned above), however, hampers their direct determination in urine unless a complicated sample purification is carried out. On the other hand, HPLC with ED is a suitable variant for the determination of DATP and DADTP in aqueous sample, whereas DAP proved to be electrochemically inactive.

The data obtained showed that HPLC with spectrophotometric detection might be successfully applied to the determination and preparative isolation of PFB derivatives of DMP, DEP, DMTP, DETP, DMDTP and DEDTP, exhibiting absorption bands at 260 nm (Fig. 3B). Fig. 3A shows a typical chromatogram of a heptane extract containing PFB derivatives of DMP (after derivatization). Applying preparative normal-phase HPLC (UV detection at 260 nm) with manual collection of

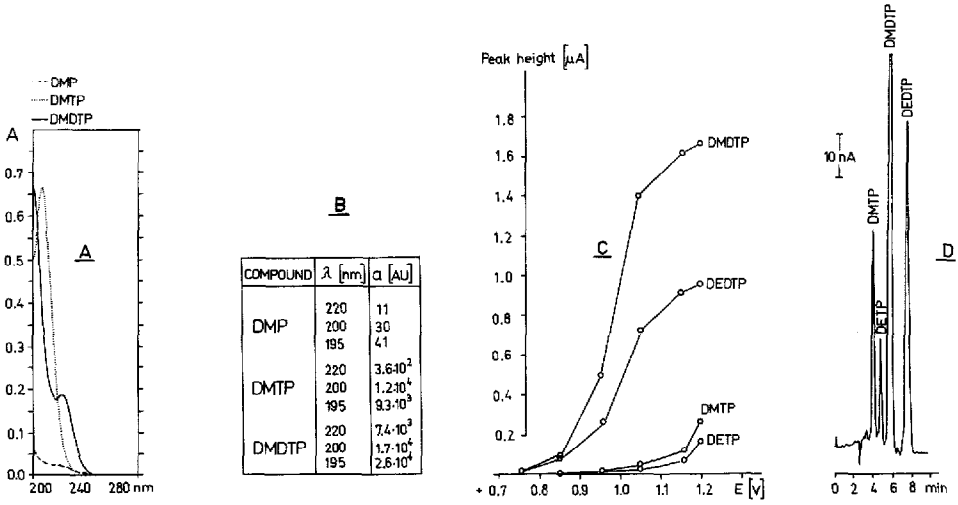

Fig. 2. Spectral, electrochemical and chromatographic characteristics of DMP, DMTP and DMDTP. (A) UV spectra (aqueous solutions), 1-cm cells; (B) molar absorbtivity data; (C) hydrodynamic voltammogram for DMTP, DETP, DMDTP and DEDTP [eluent, 0.05 *M* sodium perchlorate in 0.05 *M* phosphate buffer (pH 7.2), flow-rate 1 ml/min, glassy carbon working electrode, silver–silver chloride with 1 *M* potassium chloride reference electrode]; (D) HPLC with ED of DMTP (450 ng), DETP (450 ng), DMDTP (25 ng) and DEDTP (25 ng) using a 250 × 4.6 mm I.D. SAX (10 μm) column and mobile phase and flow-rate as in C.

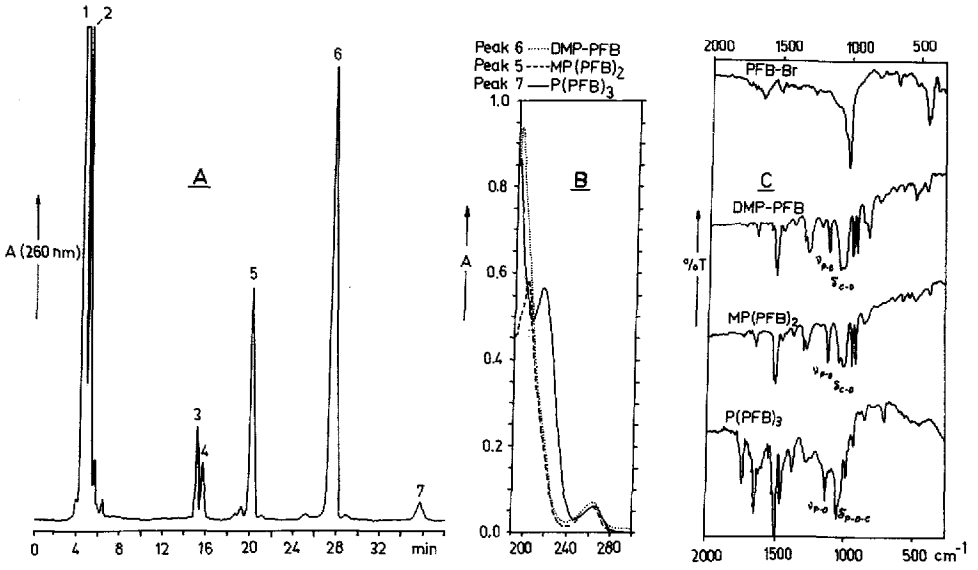

Fig. 3. (A) Preparative HPLC of heptane extract from reaction mixture (5 mg of sodium DMP + 60 μl of PFB-Br + 60 mg of potassium carbonate in 0.5 ml of acetonitrile, incubated for 3 h at 90°C); 250 × 10 mm I.D. Si-50 (7 μm) column, 2% methanol + 2% ethanol in pentane as the mobile phase at a flow-rate of 6 ml/min, UV detection at 260 n. (B) UV spectra and (C) IR spectra of the corresponding fractions.

the fractions, the compounds corresponding to peaks 1–7 were isolated. Using GC with NPD it was proved that compounds 5–7 contain phosphorous. In Figs. 3B, 3C and 4 the corresponding GC peaks and the UV and IR spectra are shown. It is evident that in the IR spectra bands due to ν_{P-O} are observed, confirming that they contain phosphorus. Similarly, the PFB derivatives of DATP and DADTP were isolated by means of preparative HPLC.

These data show that a satisfactory separation of the hydrolytic metabolites of OP pesticides is achieved using ion-exchange HPLC (or ion-pair HPLC) without preliminary derivatization. However, the absence of a specific detector highly sensitive to OP compounds hinders their direct quantitation by HPLC when present in low concentrations. This problem can only be partially solved using HPLC with ED for the determination of thio- and dithiophosphates in aqueous solution, and even in urine and other samples after purification. For this reason GC after derivatization was applied using both packed and capillary columns.

A systematic investigation of the GC separation of DMP-PFB, DEP-PFB, DMTP-PFB, DETP-PFB, DMDTP-PFB, DEDTP-PFB, $MP(PFB)_2$ and $P(PFB)_3$ (the last two are formed as side-products in the course of derivatization) using packed columns with different stationary phases was performed. SE-30, DC-200, OV-17, OV-25, OV-225, QF-1, Carbowax 20M and their binary mixtures were tested. The results showed that satisfactory separation was obtained using OV-17 (better when coated on Chromosorb G than on Chromosorb W) and much better when a column packed with a mixture of 2% OV-225 coated on Chromosorb W HP (100–120 mesh) and 3% OV-17 coated on the same support in a ratio of 1:2 was used. Fig. 4A shows a typical chromatogram obtained on this stationary phase mixture packed in

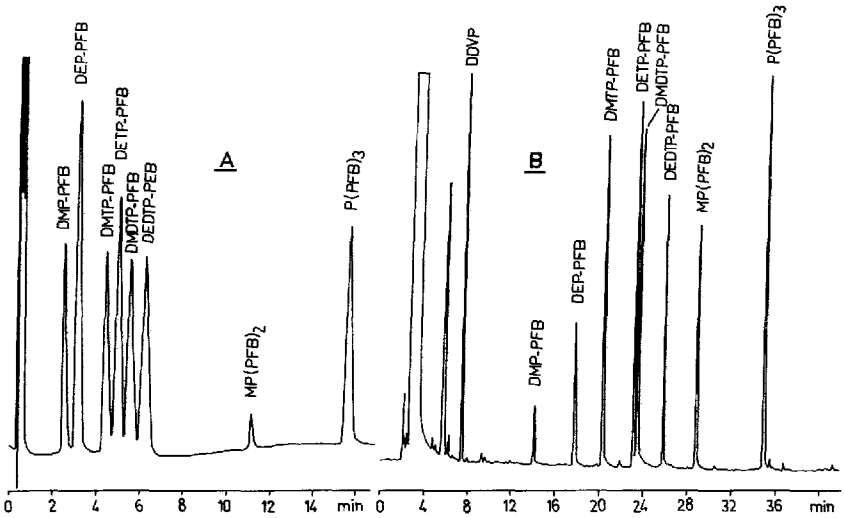

Fig. 4. GC of a heptane solution of PFB derivatives. (A) 2 m × 1.75 mm I.D. glass column packed with mixture of 2% OV-225 on Chromosorb W HP (100–120 mesh) and 3% OV-17 on the same support in the ratio 1:2; injector temperature, 240°C; column temperature, 185°C for 8 min then increased to 240°C at 20°C/min; NPD in P-mode at 280°C, hydrogen flow-rate 44 ml/min and oxygen flow-rate 32 ml/min; carrier gas, nitrogen at a flow-rate of 35 ml/min. (B) 50 m × 0.23 mm I.D. fused-silica capillary column, PVMS stationary phase; injector temperature, 240°C; column temperature programmed from 75 to 270°C in three ramps; NPD in NP mode at 250°C; carrier gas, helium at a flow-rate of 3 ml/min.

a 2 m × 1.75 mm I.D. glass column with the column temperature programmed from 185°C (8 min) to 240°C at 20°C/min. The chromatogram also indicates that under these conditions the presence of nitrogen in the stationary phase (OV-225) does not interfere and NPD can be used at column temperatures up to 240°C. It was also shown that silylation of both the column and injector (with pentafluorophenyldimethylchlorosilane as the silylating agent at 150°C) influences the efficiency (in this procedure the column should be disconnected from the detector).

Several different types of capillary columns were also checked for the separation of PFB derivatives. It was found that columns with Carbowax 20M and SP-1000 are not convenient (low temperature limit), but the column with PVMS as stationary phase proved to be suitable for the separation of the all PFB derivatives of OP compounds and also DDVP, which can be used as an internal standard (added after derivatization). The separation of DMP-PFB, DEP-PFB, DMTP-PFB, DETP-PFB, DMDTP-PFB, DEDTP-PFB, $MP(PFB)_2$, $P(PFB)_3$ and DDVP on a 50 m × 0.23 mm I.D. fused-silica capillary column is illustrated in Figs. 4B and 5. The high temperature limit of the column permits the determination of di- and tri-PFB derivatives and also the purging of higher boiling compounds (column purification) when urine samples are analysed. In the chromatograms of urine samples from persons exposed to OP pesticides, peaks with retention times in the range 25–40 min are observed (Fig. 5), but not in those of urine from non-exposed persons. Their intensities increase with increasing storage time of the samples up to 100 days after collection. They are probably due to other derivatives of OP pesticides, such as di- and/or tri-PFB thio- and dithiophosphates. Unfortunately, these peaks could not be identified as no standard substances were available.

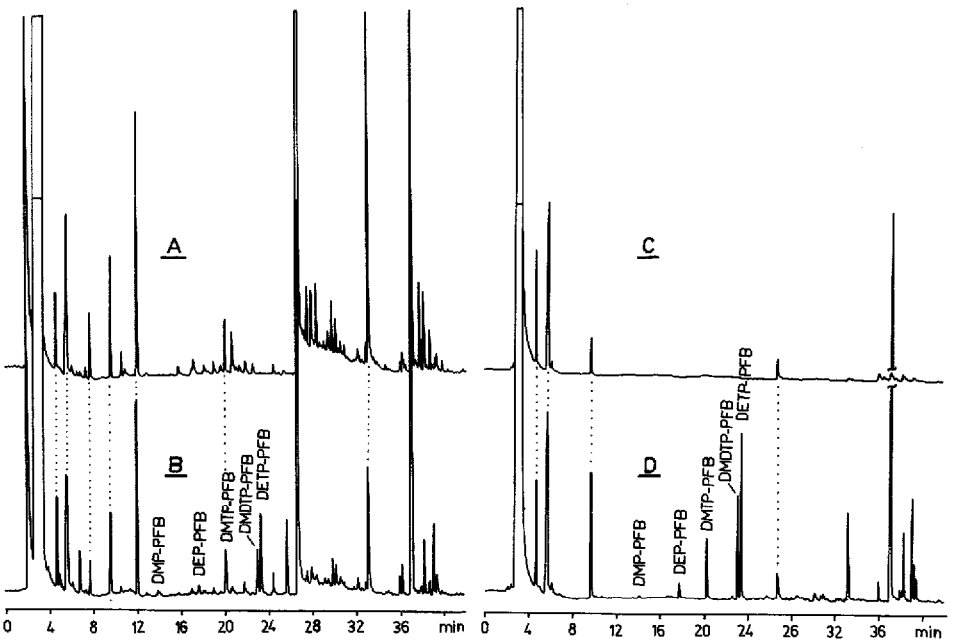

Fig. 5. GC of urine samples from non-exposed persons (A, C) and persons exposed to Supracide, Fenitrothion and Dursban (B, D). GC conditions as in Fig. 4B. (A, B) Chromatograms of heptane extracts after derivatization without acid washing; (C, D) chromatograms of the same extracts after acid washing.

TABLE I

RECOVERY DATA FOR EXTRACTIVE ALKYLATION

Ion-pair reagent	*Recovery (%)*					
	DMP	*DEP*	*DMTP*	*DETP*	*DMDTP*	*DEDTP*
Benzyltetrabutylammonium chloride (276)*, 0.1 *M* solution in 0.1 *M* NaOH	1	1	10	65	40	70
Benzyldimethylhexadecyl-ammonium chloride (360)* 0.1 *M* solution in 0.1 *M* NaOH	1	1	20	80	70	80
N-Benzyl-N,N-dimethyl-4-(1,1,3,5-tetramethylbutyl)-phenoxyethoxyethyl-ammonium chloride (412)*, 0.1 *M* aqueous solution	5	10	50	80	70	80
Tetraphenylarsonium chloride (383)*, 0.1 *M* solution in 0.1 *M* NaOH	5	5	50	80	70	80
Dicyclohexyl-18-crown-6 (411)**, 0.1 *M* solution in 0.05 *M* K_2CO_3	1	1	60	80	90	80

* Ionic mass of the corresponding cations.
** Ionic mass of the K^+ complex.

In order to optimize the conditions for the extractive alkylation according to Bradway *et al.*[12], the type of ion-pair reagent and the acidity of the aqueous phase were varied. The results obtained are given in Table I. It is evident that the procedure is not applicable to DMP and DEP as the analytical yields are low.

Using the procedure of Reid and Watts[13], derivatization of all phosphates soluble in water–acetonitrile was achieved. In this instance, however, other problems were encountered, namely different rates of esterification of different phosphates and the occurrence of side-reactions. For this reason we examined the applicability of the derivatization using other experimental conditions: (a) when the derivatization was performed in acetone and in the presence of potassium carbonate at 45°C for 4 h no side-reactions were found, but the analytical yield was still low (less than 40% for DEP and less than 30% for DMP); (b) application of a K^+–crown ether complex as a catalyst for he derivatization[14] (1 mg of crown ether and 20 mg of potassium carbonate per millilitre of reaction mixture) proved to be effective in acetonitrile medium (no catalytic effect was observed in acetone medium), the rate of derivatization for DMP and DEP increasing more than 3-fold at 90°C.

The standardization of the derivatization provides an opportunity to exclude the derivatization of the reference substances and the use of their PFB derivatives for calibration of the retention times and R_F values in the course of their quantitation. The acid washing of the extract obtained from the reaction mixture proposed before the GC determination (Fig. 4) leads to a significant improvement in the selectivity.

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