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Determination of organophosphorus and carbamate pesticide standards by liquid chromatography with detection by inhibition of immobilized acetylcholinesterase

M. E. LEON-GONZALEZ and ALAN TOWNSHEND*
School of Chemistry, University of Hull, Hull HU6 7RX (U.K.)
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ABSTRACT

A sensitive method for post-column reaction detection of carbamate and organophosphorus pesticides is described, based on the inhibition of immobilized acetylcholinesterase. The compounds are separated by reversed-phase liquid chromatography with tetrahydrofuran–water as the mobile phase in a isocratic system. The reactor used for detection comprises a conventional flow injection assembly for monitoring activity of acetylcholinesterase immobilized on controlled pore glass in a mini-column with spectrophotometric detection. The detection limits and linear calibration ranges are 2.6 and 5–40 ng for paraoxon, 0.04 and 0.1–1.6 ng for diisopropylfluorophosphate, 18 and 20–100 ng for isopropyl N-phenylcarbamate and 29 and 40–400 ng for isopropyl N-(3-chlorophenyl)carbamate. Only 16 min is required for the determination of a mixture of the above pesticides, and each enzyme column can be used for 90 determinations.

INTRODUCTION

Organophosphorus compounds are widely used in agriculture, medicine and industry, and include some highly toxic chemical warfare agents. Carbamates and organophosphorus pesticides are frequently applied in agriculture. However, whereas the organophosphorus compounds are generally used alone, they may be also be applied in conjunction with a carbamate pesticide for the control of a specific pest showing resistance to the organophosphorus compound.

Carbamates and organophosphorus pesticides have been applied to a variety of crops including green vegetables. These uses leave residues on the crops and also contaminate surface waters draining from the cropland. The presence of carbamates and organophosphorus pesticides in water, food and animal feedstuffs presents a potential hazard owing to their high mammalian toxicity. Under environmental conditions carbamates and organophosphorus pesticides could persist at the mg l^{-1} level in water, food and feed for a number of days or weeks depending on temperature and pH [1]. This makes it necessary to develop analytical methods which offer high selectivity and sensitivity for both identification and quantitation.

Ideally, multiresidue methods should provide rapid identification and quantifi-

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cation of as many different pesticides as possible in a wide array of sample matrices. Although reversed-phase liquid chromatography (LC) has often been used for analysis of carbamates, this is not the case for the organophosphorus pesticides. This situation arises because carbamate pesticides are difficult to analyse by gas chromatography because of thermal instability and that LC detectors often lack the sensitivity required for organophosphorus pesticide analysis. Pre-column derivatization for improved UV detection of pesticides in LC is always a possibility, though this requires additional sample handling, treatment, and work-up and provides additional room for error. Post-column detectors based on specific chemical reactions offer an alternative method, with excellent selectivity and sensitivity, providing that the extracolumn peak broadening is kept to a minimum.

One of the most notable changes that is occurring in analytical chemistry in general and pesticide analysis in particular is the rapidly growing impact of biotechnology. Many workers [2–9] have reported the enzymatic determination of carbamate and organophosphorus pesticides. Unfortunately, these methods are of limited used because of a lack of specificity and do not allow the simultaneous determination of several pesticides.

The inhibition of cholinesterase enzymes has been widely applied for the detection of trace amounts of organophosphorus pesticides on thin-layer chromatograms [10–13]. Ramsteiner and Hormann [14] coupled a continuous-flow analyzer to a liquid chromatograph and determined organophosphate and carbamate insecticide residues in a plum-leaf extract. Moye and Wade [15] developed a fluorimetric enzyme-inhibition detector for carbamate pesticides and applied the system to the determination of these compounds in crop samples. It should be borne in mind that these methods are suitable only for polar compounds soluble in water. Sipponen [16] developed a spectrophotometric enzyme-inhibition detector for organophosphorus compounds not detectable in trace amounts with any conventional liquid chromatography detector, and explored the possibility of using organic solvents in the mobile phase. The major fault of all these systems is the need to pump relatively large amounts of enzyme solutions in the flow systems used.

Recently, we reported the development of an enzyme-inhibition detector [9] in a flow system for the sensitive determination of paraoxon. The procedure was based on acetylcholinesterase immobilized on controlled-pore glass, and spectrophotometric monitoring of the enzyme-catalyzed reaction. The detection limit for paraoxon was $8 \cdot 10^{-9}$ *M* in a stopped flow system, and $4 \cdot 10^{-7}$ *M* in a continuously flowing system. This paper describes the development of a method for the simultaneous determination of two carbamate and two organophosphorus pesticides using reversed-phase LC coupled with a detector based on that continuous flow system involving immobilized acetylcholinesterase.

EXPERIMENTAL

Reagents

Acetylcholinesterase (E.C. 3.1.1.7, AChE, from eel, Type VI-S, 200 U mg^{-1}) and controlled porosity glass (CPG-240, 80–120 mesh, 22.6 nm mean pore diameter) were obtained from Sigma. Diethyl-*p*-nitrophenylphosphate (paraoxon, 95% pure, Sigma), isopropyl N-phenylcarbamate (99% pure, Sigma) and isopropyl N-(3-chloro-

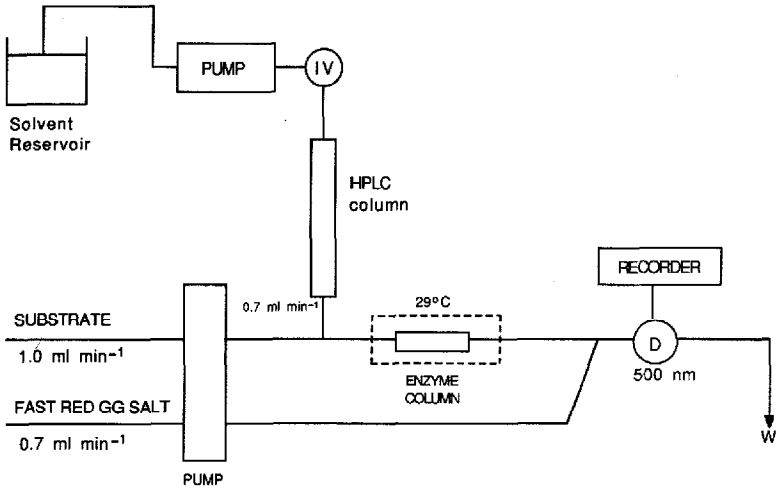

Fig. 1. Liquid chromatograph-flow detector assembly. Fast red GG salt is *p*-nitrobenzenediazonium fluoroborate; substrate is α-naphthyl acetate; enzyme is acetylcholinesterase. D = Detector; W = waste.

phenyl)carbamate (Sigma) were used for preparing 1000 mg l^{-1} stock solutions in methanol. Diisopropylfluorophosphate (DFP, Sigma) was used for preparing a 100 mg l^{-1} stock solution in methanol. No changes were observed in these solutions after 1 week at 4°C. Standard solutions of the pesticides were prepared by dilution of the stock solutions with water. A $5.0 \cdot 10^{-3}$ *M* stock solution of α-naphthyl acetate (Sigma) was prepared by dissolving 0.093 g in 100 ml of acetone–water (5:95). A $5.0 \cdot 10^{-3}$ *M* stock solution of *p*-nitrobenzenediazonium fluoroborate (Sigma) was prepared by dissolving 0.118 g in 100 ml of water.

The pH was adjusted with a 0.05 *M* phosphate buffer (pH 8.0) containing 45 m*M* sodium chloride and 12 μ*M* magnesium chloride. HPLC grade methanol, acetonitrile (May and Baker) and tetrahydrofuran (Aldrich) were used in preparing mobile phases. Distilled deionized water was used throughout.

The stock solutions were kept in a refrigerator. All chemicals used were of analytical or HPLC grade.

Enzyme immobilization

This was achieved on CPG following the procedure described earlier [9]. The product was packed in a glass tube (10 × 2.5 mm I.D.), so that the length of the immobilized enzyme zone was 10 mm. The beads were stored at 4°C in 0.05 *M* phosphate buffer, pH 6.0.

Apparatus and procedures

The liquid chromatographic system consisted of a RR/035 solvent pump (Milton Roy) equipped with a Rheodyne 7125 valve injector (20-μl loop) connected to a Spectromonitor (LDC) variable wavelength detector. A Labdata (Labdata Instrument Services Ltd.) recorder was connected to the system. Spherisorb S10 ODS2 or Spherisorb 5 Amino chromatographic columns (Phase Separations), 25 cm × 4.6 mm I.D., 10 or 5 μm particle size, respectively, were used.

The assembly of the liquid chromatograph and the spectrophotometric enzyme-inhibition detector is shown in Fig. 1. The absorbance was measured at 500 nm. The manifold and reaction coil tubing were 0.5 mm I.D. PTFE. The pump was a Gilson Minipuls 2 (Anachem). The effluent from the chromatographic column was merged with the buffered substrate solution ($3.0 \cdot 10^{-4}$ *M* α-naphthyl acetate in 0.05 *M* phosphate buffer containing 45 m*M* sodium chloride and 12 μ*M* magnesium chloride). The substrate was hydrolyzed by the immobilized enzyme to α-naphthol which reacts with *p*-nitrobenzenediazonium fluoroborate, providing a continuous baseline absorbance at 500 nm. In the presence of a cholinesterase inhibitor in the column effluent, enzyme activity is temporarily decreased in proportion to the inhibitor concentration, thus producing a decrease in absorbance (negative peak).

In order to avoid contamination, all apparatus exposed to pesticide solutions were carefully cleaned by contact with 2% (w/v) sodium hydroxide solution for a few hours to ensure hydrolysis of the pesticide.

RESULTS AND DISCUSSION

Effect of organic solvent on immobilized enzyme activity

A major problem in employing an enzyme in a LC detector is to find a mobile phase that allows analytical separation of the desired compounds but does not at the same time inactivate the enzyme and decrease sensitivity. Solvent systems of water mixed with methanol, acetonitrile or tetrahydrofuran are widely used in reversed-phase LC, and the influence of these three solvents on the activity of immobilized AChE and its inhibition was investigated.

This study was carried out using the flow injection procedure described earlier [9], except that the carrier solution contained up to 40% (v/v) organic solvent. Significantly higher concentrations of organic solvent completely inactivated the

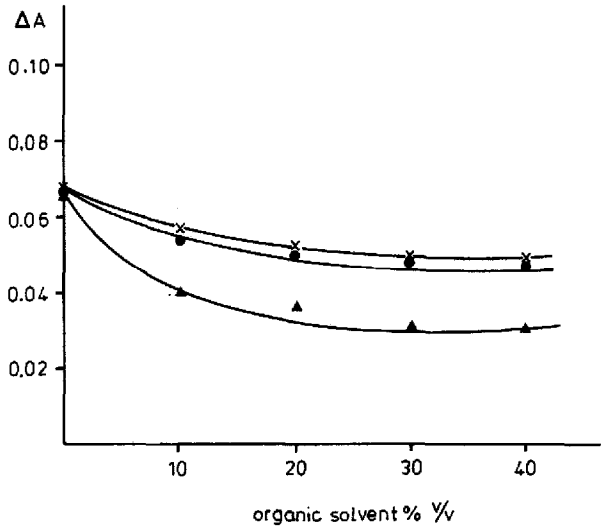

Fig. 2. Effect of organic solvent content on inhibition of immobilized acetylcholinesterase by paraoxon: × = methanol–water; ● = tetrahydrofuran–water; ▲ = acetonitrile–water.

enzyme. Up to 40% organic solvent had little effect on the baseline absorbance, but above 20% there is an appreciable increase in detector noise. Sipponen [16] reported that 8% acetonitrile totally inhibited soluble electric eel AChE. The same author reported that up to 20% methanol appeared to increase the activity of butyrylcholinesterase. Neither effect was observed in this work for the immobilized AChE.

The results obtained for inhibition by paraoxon are shown in Fig. 2. The peak height for $8.0 \cdot 10^{-6}$ *M* paraoxon decreases steadily in all organic solvents, but most appreciably in acetonitrile. Thus methanol or tetrahydrofuran would be more satisfactory solvents if high sensitivity is to be achieved. Reproducible responses to pesticides were achieved when the proportion of methanol or tetrahydrofuran in the reaction mixture did not exceed 20%. Thus, in subsequent experiments the flow-rates were adjusted to ensure that the concentration or organic solvent in the solution pumped to the enzyme was about 20%.

Selection of chromatographic conditions

Efficient separation conditions were established by using reversed-phase LC with UV detection at 254 nm. A Spherisorb S10 ODS2 or Spherisorb 5 Amino column was used with isocratic elution by 25, 40, 50, 75 or 90% organic solvent in the mobile phase. The best separations achieved are shown in Figs. 3 and 4. As would be expected, DFP solutions at concentrations as high as 200 mg l^{-1} could not be detected by UV spectrophotometry at 254 nm.

The chromatographic conditions used in Figs. 3 and 4 were chosen to study the possibility of combining the LC system with the enzymatic inhibition detection system. Although separation on Spherisorb 5 Amino with methanol–water (40:60) mobile

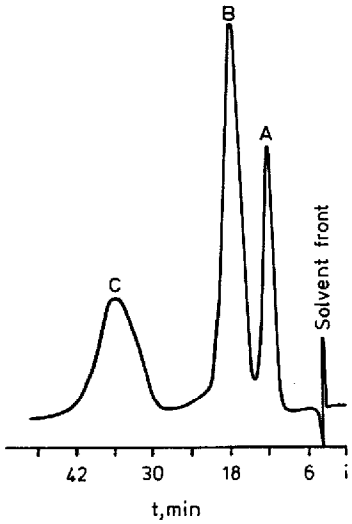

Fig. 3. Chromatogram showing the simultaneous separation of paraoxon and carbamate pesticides by isocratic elution. Column: Spherisorb 5 Amino. Mobile phase: methanol–water (40:60). Flow-rate: 1.0 ml min^{-1}. UV detection at 254 nm. Solute concentrations: 50 μg ml^{-1}. Peaks: A = isopropyl N-phenylcarbamate; B = paraoxon; C = isopropyl N-(3-chlorophenyl)carbamate. i = Injection.

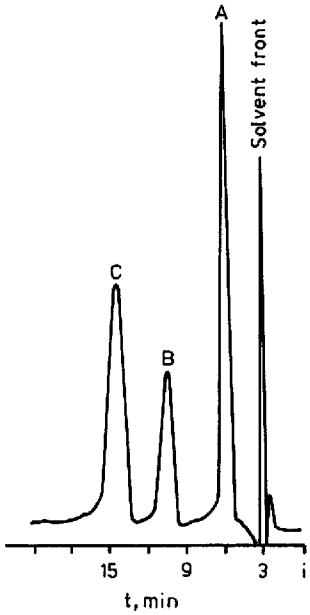

Fig. 4. Chromatogram showing the simultaneous separation of paraoxon and carbamate pesticides by isocratic elution. Column: Spherisorb S10 ODS2. Mobile phase: tetrahydrofuran–water (50:50). Flow-rate: 0.7 ml min^{-1}. UV detection at 254 nm. Solute concentrations: 10 μg ml^{-1}. Peaks: A = paraoxon; B = isopropyl N-phenylcarbamate; C = isopropyl N-(3-chlorophenyl)carbamate.

phase gave more sensitivity, this system was rejected because DFP has a retention time of 55 min under these conditions. Therefore, separation with tetrahydrofuran–water (50:50) on Spherisorb S10 ODS2 was chosen for further studies. Fig. 5 shows representative chromatograms of a four-pesticide sample obtained by using UV or enzymatic detection. The separation was achieved in 16 min. The enzymatic detector allows detection of traces of DFP, unlike the UV detector.

High sensitivity and good repeatability are achieved by keeping the concentration of tetrahydrofuran that passed through the enzyme column at *ca.* 20%. This amount is enough to keep in solution the α-naphthol produced by the enzymatic hydrolysis. It was found, however, that the presence of tetrahydrofuran decreases the useful life of the column; it was possible to make about 90 determinations of pesticides instead of the 150 reported earlier before significant loss of sensitivity occurred [9]. A change of enzyme column every 90 determinations is therefore recommended.

Analytical characteristics

Linear relationships between peak height and concentration were obtained in the range 5–40 ng for paraoxon, 0.1–1.6 ng for DFP, 20–100 ng for isopropyl N-phenylcarbamate and 40–400 ng for isopropyl N-(3-chlorophenyl)carbamate. The detection limits (amounts giving signal-to-noise ratio of 2) were 2.6 ng of paraoxon, 0.04 ng of DFP, 18 ng of isopropyl N-phenylcarbamate and 29 ng of isopropyl N-(3-chlorophenyl)carbamate. The detection limits for UV detection were 18, 23 and

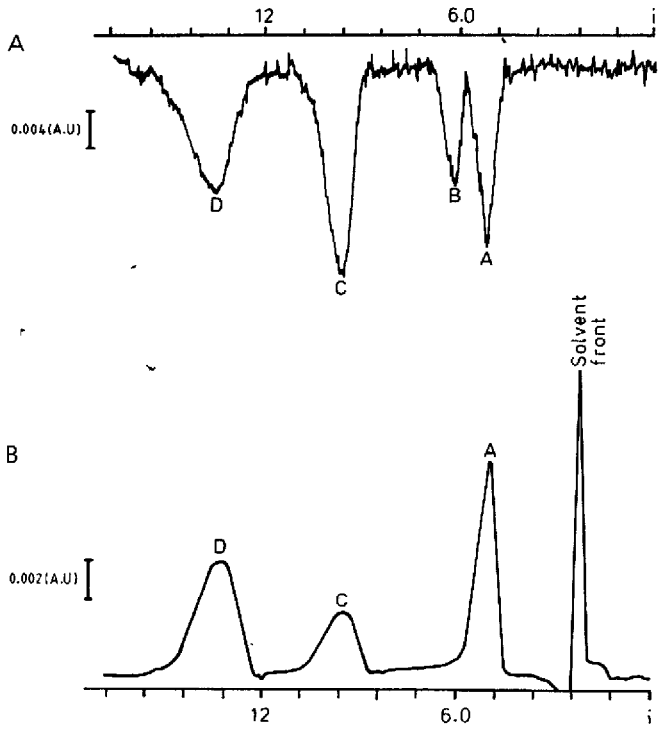

Fig. 5. Comparison of chromatograms of carbamate and organophosphorus pesticides. Column: Spherisorb S10 ODS2. Mobile phase: tetrahydrofuran–water (50:50). Flow-rate: 0.7 ml min^{-1}. Peaks: A = paraoxon; B = DFP; C = isopropyl N-phenylcarbamate; D = isopropyl N-(3-chlorophenyl)-carbamate. (A) Enzymatic detection; solute concentrations: 2 mg ml^{-1} for A, C, D, 0.04 mg ml^{-1} for B. (B) UV detection, solute concentrations: 20 mg ml^{-1}.

40 ng, respectively, which compare well with those reported by Lawrence and Turton [17]. Relative standard deviations ($n = 6$) were 1.2% (20 ng of paraoxon), 1.5% (0.2 ng of DFP), 1.7% (30 ng of isopropyl N-phenylcarbamate) and 1.9% [60 ng of isopropyl N-(chlorophenyl)carbamate].

The repeatability of retention times was studied by injecting 20 ng of paraoxon, 0.6 ng of DFP, 30 ng of isopropyl N-phenylcarbamate and 40 ng of isopropyl N-(3-chlorophenyl)carbamate on four successive days. The relative standard deviation of the retention times was 1.2%. Only 16 min were required for each complete determination.

CONCLUSIONS

Immobilized acetylcholinesterase can successfully be utilized in a post-column reactor system for detection of traces of organophosphorus and carbamate pesticides. The arrangement described allows the use of eluents containing organic solvents. High sensitivity and good repeatability are achieved by restricting the amount of the organic solvent to about 20%. Direct injection of sample is possible without the problems of

non-volatile compounds which are significant in gas chromatographic analyses. Mixtures of carbamates and organophosphorus pesticides can be analysed without changing the column or detector.

The proposed system should be applicable to the detection of any cholinesterase inhibitor. By using enzymes other than cholinesterase in an immobilized form, continuous assay of other compounds that are potent enzyme inhibitors should also be possible.

Sensitivity of detection depends crucially on the ability of the compound to inhibit the particular enzyme preparation. Several reports [5–8,11,12,16] have shown that organophosphorus and carbamate compounds tend to differ markedly in their ability to inhibit cholinesterases from different sources. Therefore, studies of cholinesterases from different sources could provide more sensitivity for certain cholinesterase inhibitors.

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