Journal of Chromatography, 389 (1987) 87–94
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 171

DETECTOR FOR ORGANOPHOSPHORUS COMPOUNDS IN LIQUID CHROMATOGRAPHY BASED ON THE CHOLINESTERASE INHIBITION REACTION

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(Received September 15th, 1986)

SUMMARY

A sensitive method for the post-column reaction detection of organophosphorus compounds is described. The method relies on cholinesterase and is particularly suitable for the analysis of potent inhibitors such as sarin, soman and tabun. The compounds are separated by reversed-phase chromatography with methanol–water as the mobile phase in a linear gradient system. The reactor used for the detection comprises conventional autoanalyzer equipment with air segmentation of the reactor stream. The detection limits are 10 pg for sarin and soman and 60 pg for tabun. A quantitation method is presented, based on the linear correlation between the residual enzyme activity and the inhibitor concentration. The repeatability is ± 1%. As a test of the system, the model compounds were detected against a background of urban air.

INTRODUCTION

Analytical methods for organophosphorus (OP) compounds are of great interest because of the extensive use of these compounds in agriculture, medicine and industry. Also included among OP compounds are some highly toxic chemical warfare agents. Liquid chromatography (LC) methods for trace analysis of OP pesticides and related compounds have often suffered from the lack of sufficiently selective and sensitive detectors. The UV detector, which is widely used for residue determinations[1,2], cannot be used for those OP compounds that lack suitable chromophores. Moreover, sample matrices typically contain several UV-absorbing compounds, and time-consuming sample preparation or clean-up may be required before analysis. 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.

The inhibition of cholinesterase enzymes has been widely applied for the detection of trace amounts of OP pesticides from thin-layer chromatograms[3–7]. Ramsteiner and Hörmann[8] coupled a continuous-flow analyzer to a liquid chromatograph and determined organophosphate and carbamate insecticide residues in a plum leaf

0021-9673/87/$03.50

extract. Moye and Wade[9] developed a fluorometric enzyme-inhibition detector for carbamate pesticides and applied the system to the analysis of these compounds in crop samples. The major problem in employing enzymes is to find a mobile phase that allows analytical separation of the desired compounds but does not at the same time inactivate the enzyme.

The goal of this work was to design an enzymatic LC reactor system for the detection of trace amounts of OP compounds in environmental samples. The model compounds chosen for the study were ones not detectable in trace amounts with any conventional LC detector. In particular, the possibility was explored of using organic solvents in the mobile phase. After selection of a suitable mobile phase and the elution mode, detection limits were determined for the compounds. A quantitation method was developed and the repeatability and linearity of response were calculated. Electric eel acetylcholinesterase (AChE) and human serum pseudocholinesterase (ChE) were used as enzymes. The usefulness of the system was verified by a detection of the model compounds in urban air. An UV detector was used on-line with the enzymatic detection system to provide comparative information about the UV-absorbing compounds present in the urban environment.

EXPERIMENTAL

Materials and methods

Electric eel AChE (Type VS) was obtained from Sigma and human serum ChE was obtained from the Research Center of the Finnish Defense Forces. The lyophilized preparations were dissolved in 52 m*M* phosphate buffer, pH 7.2, containing 0.1 *M* sodium chloride and 0.1% gelatine (PBSG). These enzyme solutions retained their activities for at least 2 months when stored at +4°C. Enzymatic determinations were performed according to the method of Ellman *et al.*[10]. The colour reagent, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma), was dissolved in PBSG at a concentration of 0.26 m*M*. This solution is stable for several weeks when stored at +4°C and protected from light. Immediately before use the enzyme preparations were diluted in the DTNB–PBSG solution. The substrate was acetylthiocholine iodide (puriss., Fluka). This was dissolved in distilled water at a concentration of 100 m*M*, and the solution was divided into 1-ml aliquots and frozen at −25°C. The frozen substrate solution can be stored for at least 3 weeks in the dark. Before use the substrate was diluted 1:10 in distilled water.

The three model compounds, isopropyl methylphosphonofluoridate (sarin), pinacolyl methylphosphonofluoridate (soman) and ethyl N,N-dimethylphosphoroamidocyanidate (tabun), were obtained from the Research Center of the Finnish Defense Forces. According to ^{19}F and ^{31}P nuclear magnetic resonance (NMR) spectrometry, the compounds were 70–85% pure. The diluting solvent was acetone or diethyl ether, and concentrations were determined with a gas chromatograph equipped with an alkali thermionic detector and with tri-*n*-butyl phosphonate and diisopropyl methylphosphonate added as internal standards. The structures of the compounds studied are presented in Fig. 1. The components of the mobile phase in LC were methanol (Merck) and distilled water. Before use the solvents were filtered, using a Millipore apparatus, through a 0.5-μm membrane filter.

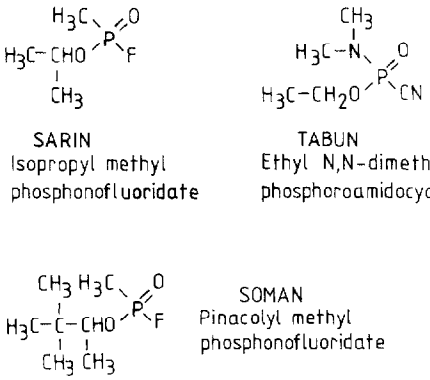

Fig. 1. Structures of sarin, soman and tabun.

Equipment

The high-performance liquid chromatographic (HPLC) system consisted of a Kontron Model 640 chromatograph equipped with a Kontron programmer Model 200 and a Kontron Uvikon 722 LC variable-wavelength detector (190–900 nm). A two-channel Servogor 120 recorder was connected to the system. A LiChrosorb Hi-bar RP-18 column from Merck, 250 mm × 4.0 mm I.D., 5-μm particles, was used throughout the study.

The assembly of the liquid chromatograph and reaction detector is shown in Fig. 2. The continuous-flow detector (λ = 405 nm) was constructed from the automatic chemical analysis system (AKEA) produced by Datex (Instrumentarium Oy, Finland). The analyzer was equipped with two thermostatted water-baths and the reaction coils located in these baths were thermostatted at 37°C. A multi-channel peristaltic pump was used for supplying the reagents, and air was pumped in for segmentation of the reaction stream. The reaction coils, mixing coils, connectors and fittings were obtained from Instrumentarium Oy. Pump tubes and manifold tubing, with inner diameters as shown in Fig. 2, were Technicon™ standard tubings (Solvaflex). The inhibition period (during which the effluent is incubated with the enzymes) was 14 min and the colour reaction period (after addition of substrate) was 3 min.

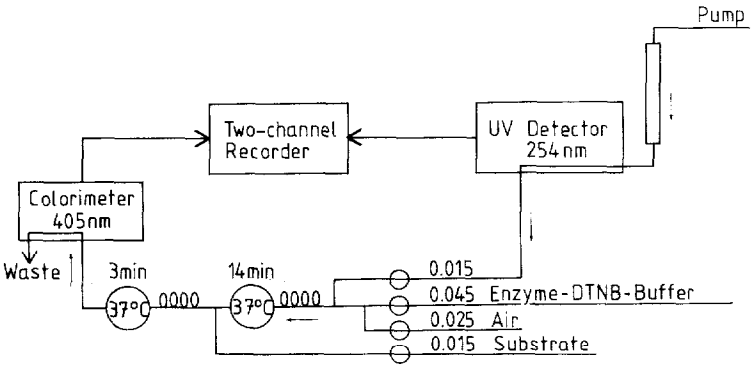

Fig. 2. The liquid chromatograph–reaction detector assembly.

The UV detector used was placed between the outlet of the column and the reaction detector. The effluent was first monitored with the UV detector (λ = 254 nm), after which a portion of it was conducted via a split to the autoanalyzer for recording of the enzyme inhibitor peaks.

Analysis procedure for air samples

Air samples were collected on the roof of a six-storey building in downtown Helsinki. The sampling time was about 24 h and the volumes of the air samples varied from 1100 to 1600 m^3. The samples were spiked by applying a solution containing 4.1 μg sarin, 3.5 μg soman and 3.6 μg tabun evenly around the filter part of the sampling apparatus at the beginning of the sampling period. Background samples were collected to check for the existence of pesticides or other compounds having an inhibitory effect on cholinesterases. After sampling, the resin (XAD-2) was removed to a Soxhlet apparatus and extracted for 5–9.5 h with about 700 ml diethyl ether. Preconcentration to a volume of 1–10 ml was carried out in a Kuderna-Danish apparatus. Finally the samples were carefully concentrated to about 500 μl under a mild stream of nitrogen. A 7-μl volume of the concentrated sample was injected into the chromatograph. The high-volume air sampler, sampling procedures and sample pretreatment have been described in detail elsewhere[11].

RESULTS AND DISCUSSION

Choice of the mobile phase

Solvent systems of water mixed with methanol or acetonitrile are widely used in reversed-phase HPLC, and the influence of these two solvents on the activities of

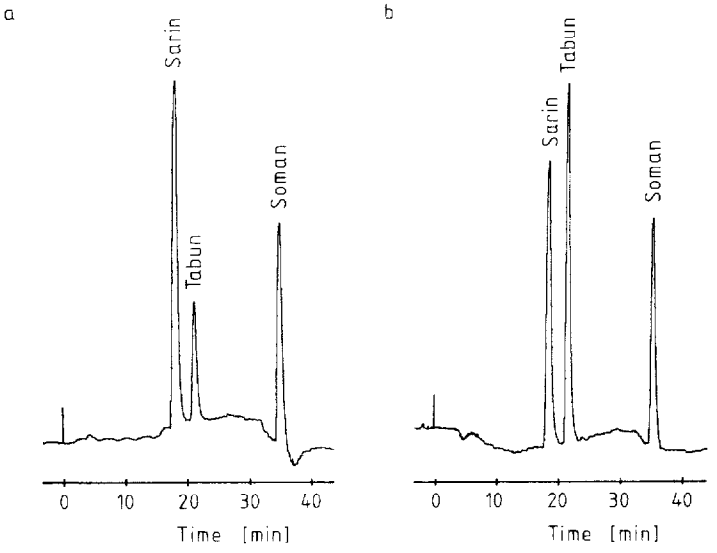

Fig. 3. Detection of sarin, soman and tabun in acetone using electric eel AChE (a) and human serum ChE (b). Conditions: linear gradient from 15 to 65% methanol in water in 35 min, flow-rate 0.7 ml/min; column, LiChrosorb RP-18 (5 μm), 250 mm × 4.0 mm.

cholinesterases was tested. The activities of both electric eel AChE and human serum ChE were decreased more by acetonitrile than by methanol, and the latter was accordingly chosen as the solvent component of the mobile phase. A good repeatability was achieved when the percentage of methanol in the reaction mixture did not exceed 8%. Acetontrile inhibited electric eel AChE totally at this level. Up to 20% methanol appeared to increase the activity of human serum ChE. The activating influence of alcohols on cholinesterase enzyme has been reported earlier[12]. Amounts of methanol greater than 8% weakened the sensitivity and caused an increase in detector noise, especially in the case of electric eel AChE, which appeared to be more sensitive to solvent effects than was human serum ChE. In the experiments, one seventh of the effluent was pumped to the autoanalyzer and thus the amount of methanol was less than 8%.

The resolution obtained with isocratic elution programs in which the percentage of methanol in the mobile phase was 40, 50, 60 and 70% was not satisfactory. Of the several gradient programs tested, the best resolution was obtained with linear gradient elution starting at 15% methanol–water, stepping, up to 65% methanol in water in 35 min (Fig. 3) and maintaining this percentage for 3 min. The retention time of sarin appeared to be 3.5 min shorter when the solvent for the sample was diethyl ether than when it was acetone.

Analytical aspects

The detection limits were determined as the amount that gave a detector response equal to twice the magnitude of the background noise. The detection limits were 10 pg for sarin and soman when electric eel was used and 60 pg for tabun when human serum ChE was used (Table I). The sensitivity of detection depends crucially on the ability of the compound to inhibit the particular enzyme preparation. My investigations[13], as well as the results obtained by Leegwater and Gend[14], have shown there to be great differences in the inhibition abilities of OP compounds against cholinesterase enzymes from different animal sources. An increase in the incubation time of the solute with the enzyme also improves the sensitivity. An increase in the residence time of the solute in the reactor, on the other hand, causes peak broadening, and a compromise has to be made. In this study, a small sacrifice of peak shape was made, as a long incubation time was desired to obtain maximum sensitivity of the assay.

TABLE I

DETECTION LIMITS AND THE RANGES OF LINEARITY FOR SARIN, SOMAN AND TABUN

Conditions: a linear gradient from 15 to 65% methanol–water in 35 min at a flow-rate of 0.7 ml/min; column, LiChrosorb RP-18 (5 μm), 250 mm × 4.0 mm.

Compound	*Electric eel AChE*		*Human serum ChE*	
	Detection limit (ng)	*Range of linearity (ng)*	*Detection limit (ng)*	*Range of linearity (ng)*
Sarin	0.01	0.01–0.5	0.02	0.02–1.0
Soman	0.01	0.01–0.5	0.01	0.01–0.5
Tabun	0.2	0.2–11.0	0.06	0.06–3.0

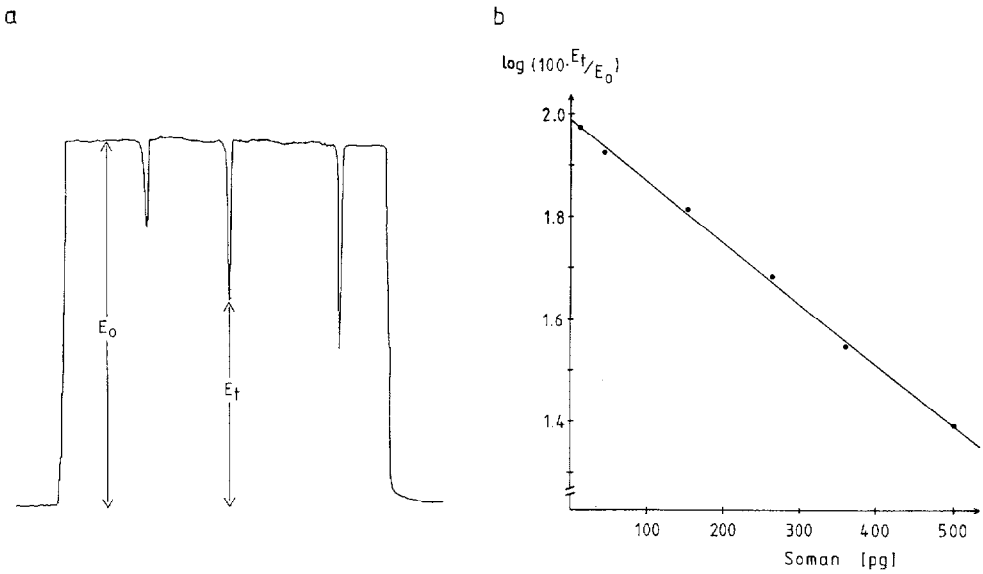

Fig. 4. (a). Determination of the initial enzyme activity, E_0, and the enzyme activity after incubation, E_t. (b) A standard plot for soman obtained with electric eel AChE.

Linearity studies of response were made under conditions where the inhibitor was in large excess, *i.e.*, where the reaction between the enzyme and inhibitor follows first-order kinetics and can be described by the equation[15,16]

$$2.303 \log (100 \cdot E_t/E_0) = -k_i I t \quad (1)$$

where E_0 and E_t are the enzyme activities before and after the incubation period, t, respectively, k_i is the rate constant and I is the inhibitor concentration. The plot of $\log (100 \cdot E_t/E_0)$ *vs.* I is linear with a slope of $-1/2.303 k_i t$. The absorbance difference between the baseline and the line obtained with the uninhibited enzyme is described by E_0 and the difference between the baseline and peak top is described by E_t (Fig. 4a). A plot of $\log (100 \cdot E_t/E_0)$ *vs.* inhibitor concentration for soman is presented in Fig. 4b. The standard curves obtained are suitable for processing by a computer system, which will determine the concentration automatically. An excellent linearity ($r = 0.998$) was obtained when the residual enzyme activities ($100 \cdot E_t/E_0$) were between 25 and 95%. The ranges of linearity of response are presented in Table I.

The repeatability, studied by repeated injections of 70 pg and 3.5 ng of sarin into the column (corresponding to the detected amounts of 10 pg and 500 pg respectively) ($n = 8$), was ±1%. Repeatability of retention times was studied by injecting sarin, soman and tabun into the column on four successive days. The relative standard deviation was ±1% ($n = 12$).

Analysis of air samples

The chromatograms obtained from the background and the spiked sample concentrates of the resin are shown in Fig. 5a and b respectively. Sarin, soman and

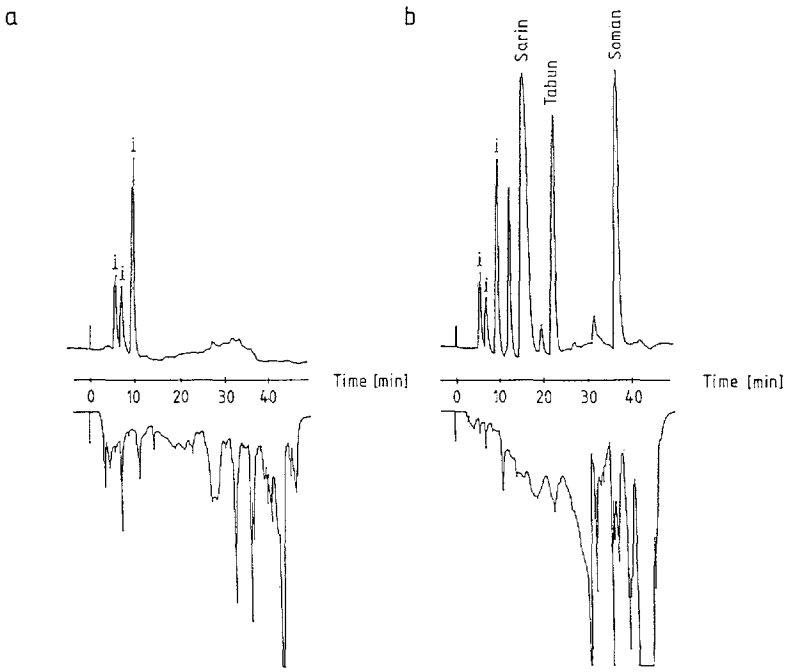

Fig. 5. Chromatograms obtained from the background (a) and spiked (b) diethyl ether concentrates of the XAD-2 resin extract. I = Impurity present in the diethyl ether concentrate. Conditions: linear gradient from 15 to 65% methanol–water in 35 min; flow-rate 0.7 ml/min; column, LiChrosorb RP-18 (5 μm), 250 mm × 4.0 mm; enzymatic detection (upper traces) with human serum ChE; UV detection (lower traces) at 254 nm.

tabun are clearly detectable in the spiked sample. The detection limits of the compounds in air were the same as those obtained for the pure compounds (Table I), demonstrating the excellent selectivity of the detector. The very large number of UV-absorbing compounds present in air is clearly indicated by the heavily loaded UV chromatograms. The three peaks present in both the background and spiked samples proved to be impurities in concentrated diethyl ether, which was twice distilled. The presence of cholinesterase inhibitors in organic solvents has been reported earlier: Vicedo *et al.*[17], for example, found phthalates and OP compounds showing cholinesterase-inhibiting activity in the distillation residue of hexane and other industrial solvents. The identity of the solvent impurities and other inhibiting compounds was not determined.

CONCLUSIONS

Cholinesterase enzymes can successfully be utilized in a post-column reactor system for detection of trace amounts of OP compounds. The arrangement described also allows the use of eluents containing organic solvents. High sensitivity and good repeatability are achieved by keeping the amount of the organic component small. The application to air samples clearly shows the effectiveness of cholinesterases for the selective detection of OP compounds. By contrast, UV chromatograms of en-

vironmental samples tend to be heavily loaded and often provide only a tentative characterization. Although peak broadening cannot be avoided, the selectivity and specificity gained in the present system clearly outweigh the loss in peak shape.

ACKNOWLEDGEMENTS

The financial support provided by the Ministry for Foreign Affairs of Finland and the Emil Aaltonen Foundation is greatly appreciated.

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