Journal of Chromatography, 100 (1974) 171–174

CHROM. 7569

GAS CHROMATOGRAPHIC DETERMINATION OF CHOLINESTERASE INHIBITION

JOZEF KOVÁČ
Research Institute of Agrochemical Technology, Bratislava-Predmestie (Czechoslovakia)
and
VALÉRIA MARKOVÁ and PETER KRÁLIK
Drug Research Institute, Bratislava (Czechoslovakia)
(Received May 14th, 1974)

SUMMARY

A new enzymatic technique for toxicological study of the inhibition of cholinesterase activity has been developed. The amount of 2,4,5-trichlorophenyl acetate (used as substrate for the esterase) and/or of 2,4,5-trichlorophenol (the product of enzymatic hydrolysis) is determined by gas chromatography, with use of an electron-capture detector selective for organochlorine compounds. This method makes possible the use of small volumes (0.1 ml) to enhance the sensitivity of trace analysis for various pesticides in toxicological work.

INTRODUCTION

The application of enzymatic techniques in trace analysis and an enzymatic detection of organophosphorus, carbamate and organochlorine insecticides in thin-layer and paper chromatography has led to substantial progress in toxicological analysis[1–6]. Various methods that have been used for determining unknown amounts of inhibitors include potentiometric[7], titrimetric[8,9], manometric[10], colorimetric[11,12], fluorescence[13] and agar-diffusion techniques[14]. Different substrates for esterases are used in these techniques[15–17]; in this work, 2,4,5-trichlorophenyl acetate (TChPA) was used. The reason for this choice was that gas–liquid chromatography (GLC) was used for the final evaluation with an electron-capture detector selective for organochlorine compounds. The technique permits detection of nanogram amounts of TChPA as well as of the product of enzymatic hydrolysis, 2,4,5-trichlorophenol (TChP). In this way, the sensitivity of the enzymatic method was enhanced.

EXPERIMENTAL

Reagents

Solvents and reagents were of analytical grade. The beef liver homogenate was prepared as described by Ackermann[3]. The solution of TChPA (500 μg/ml) was

prepared in ethanol, that of fenitrothion (10 μg/ml) was prepared in benzene, and the oxidation mixture used was glacial acetic acid–30% hydrogen peroxide (5:1).

Gas chromatography

A Hewlett–Packard research chromatograph (model 5750 G) with a Hewlett–Packard nickel-63 electron-capture detector (model 2-6195), and equipped with a Hewlett–Packard strip-chart recorder (model 7128 A), was used. The glass column (1.8 m × 3 mm I.D.) was packed with 3% of OV-17 on Chromosorb W HP (80–100 mesh). The injector block was maintained at 205°, the column at 190° and the detector at 250°. The flow-rate of the carrier gas (argon) was 50 ml/min. The detector was operated at pulse 5 and the electrometer setting was kept continuously at range 1, attenuation 64. The injection volume was 5 μl.

Determination of cholinesterase activity by GLC

Transfer different volumes of beef-liver homogenate (0.0, 0.025, 0.05, 0.10 and 0.2 ml), by pipette, into tubes, dilute each to 2 ml with citrate buffer solution of pH 4.9, and add 0.1 ml of ethanolic TChPA as substrate. Shake each solution for 15 min at room temperature, then add 5 ml of benzene, mix thoroughly, and shake for 10 min. Dilute 1 volume of the benzene layer with 20 volumes of *n*-heptane, and estimate the content of unhydrolysed TChPA by using the standard solution.

Study of inhibition of cholinesterase activity by using activated fenitrothion and GLC

The fenitrothion was activated by the following modification of the technique of Miskus and co-workers[18,19].

Add oxidation mixture (0.3 ml) to 0.5 ml of diluted benzene solutions with different concentrations of fenitrothion in glass tubes fitted with stopcocks. Mix the contents, and heat the closed tubes in a water-bath at 75 ± 1° for 20 min. Add 0.5 ml of water to the cooled solution, mix the contents of the tube, and, by pipette, introduce 0.1 ml of the separated benzene layer into another tube; in this tube place 0.01 ml of 10% (v/v) aqueous glycerine, and evaporate the benzene in a stream of air on the water-bath at 40°. To the residue add 2 ml of liver homogenate (suitably diluted with citrate buffer solution of pH 4.9), and incubate for 60 min at 37°. After cooling to room temperature, add 0.1 ml of the ethanolic TChPA, shake for 15 min, add 5 ml of benzene, mix, and continue as described for the determination of cholinesterase activity. Measure the amount of TChPA by the GLC method (value *X*).

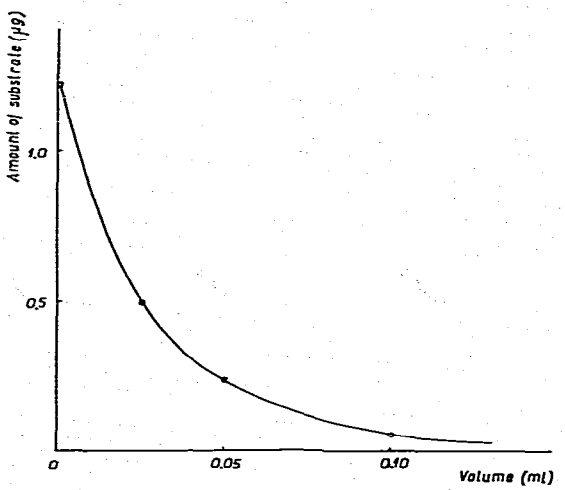
Place 0.1 ml of the TChPA solution in a tube containing 2 ml of the citrate buffer solution, and continue as described for diluted liver homogenate; the GLC estimation gives value *B*. Repeat this procedure with 0.1 ml of TChPA solution added to 2 ml of suitably diluted enzyme; this gives value *C*.

The percentage of inhibition is then given by

$$\left(1 - \frac{B - X}{B - C}\right) \times 100$$

RESULTS AND DISCUSSION

The dependence of the cholinesterase activity on the added volume of liver homogenate is shown in Fig. 1; the amount of concentrate added should be so chosen



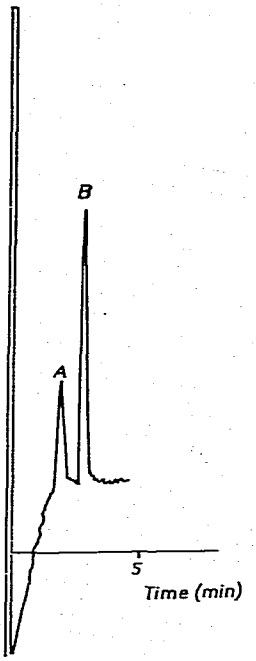


Fig. 1. Influence of added liver homogenate on unhydrolysed TChPA.

Fig. 2. GLC chromatogram of a mixture of 0.44 ng of TChP (peak A) and 1.15 ng of TChPA (peak B).

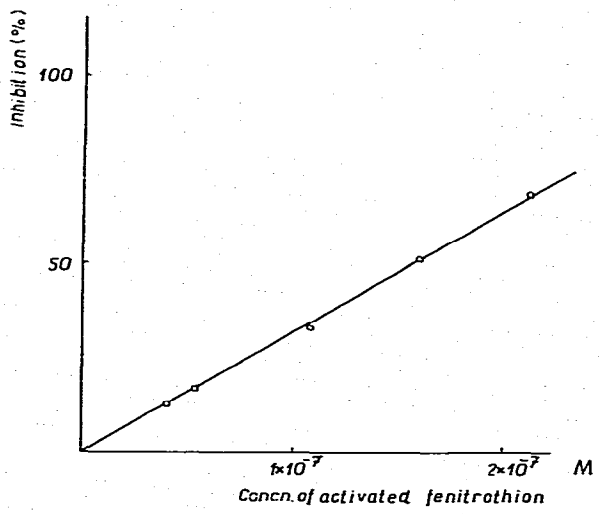


Fig. 3. Graph showing inhibition of activity of beef-liver esterase by activated fenitrothion.

that about 10–20% of unhydrolysed TChPA remains after enzymatic hydrolysis for 15 min.

The inhibition of cholinesterase activity can be evaluated either by determination of unhydrolysed TChPA or of TChP (the product of the hydrolysis): this can be seen from the resolved peaks on the chromatogram shown in Fig. 2.

It was found that hydrolysis of TChPA in aqueous media of pH 3 and 7 took place to a marked extent during the 15-min reaction period. This study was undertaken to ascertain the best buffer solution in which negligible hydrolysis was detected. For this reason, citrate buffer solution of pH 4.9 was chosen.

The linearity of the relationship between inhibition and concentration of activated fenitrothion is shown in Fig. 3: the values of increments of the inhibition with concentration ($\Delta I/\Delta c$) show a decrease at higher concentrations of inhibitor. Determinations in this range are not suitable for enzymatic analysis.

The volumes of liver homogenate, TChPA solution and benzene given in the procedure for the enzyme-inhibition study can be substantially reduced, and thus the sensitivity of the enzymatic method can be proportionally enhanced.

This method may find application in micro-toxicological studies of cholinesterase inhibition, *e.g.*, in the study of insect resistance to some insecticides.

REFERENCES

1 M. Bogusz and T. Borkowski, *Analyst (London)*, 98 (1973) 153.
2 W. P. McKinley and S. I. Read, *J. Ass. Offic. Agr. Chem.*, 45 (1962) 467.
3 H. Ackermann, *Nahrung*, 10 (1966) 273.
4 C. E. Mendoza and J. B. Shields, *J. Agr. Food Chem.*, 21 (1973) 178.
5 G. G. Guilbault, M. H. Sadar, S. S. Kuan and D. Casey, *Anal. Chim. Acta*, 52 (1970) 75.
6 H. Ackermann, *J. Chromatogr.*, 36 (1968) 309.
7 P. A. Giang and S. A. Hall, *Anal. Chem.*, 23 (1951) 1830.
8 J. C. Gage, *Advan. Pest Control Res.*, 4 (1961) 183.
9 M. S. Schechter and I. Hornstein, *Advan. Pest Control Res.*, 1 (1957) 353.
10 K. P. DuBois, in F. W. Sunderman (Editor), *Lab. Diagn. Dis. Caused Toxic Agents. Proc. Appl. Seminar Ass. Clin. Sci., Chicago*, Green, St. Louis, 1970, p. 187–189.
11 J. W. Cook, *J. Ass. Offic. Anal. Chem.*, 37 (1954) 561.
12 G. D. Winter, *Ann. N.Y. Acad. Sci.*, 87 (1960) 875.
13 G. G. Guilbault and D. N. Kramer, *Anal. Chem.*, 37 (1965) 1675.
14 E. Sandi, *Nahrung*, 6 (1962) 57.
15 W. Ebing, *J. Chromatogr.*, 42 (1969) 140.
16 R. I. Volkova, *Biokhimiya*, 32 (1969) 1253.
17 M. Mitchard, *J. Pharm. Pharmacol.*, 24 (1972) 328.
18 R. Miskus, M. E. Tzanakakis and S. M. Smith, *J. Econ. Entomol.*, 52 (1959) 76.
19 R. Miskus and S. Hassan, *J. Econ. Entomol.*, 52 (1959) 353.