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Note

Sensitive and rapid detection of paraoxon by thin-layer chromatography and strips using enzyme inhibition and Ellman's method

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Thin-layer chromatography (TLC) in combination with enzyme inhibition (EI) has been widely used in the detection of organophosphorus, carbamate and organochlorine pesticides as described in review and survey articles by Mendoza[1–3] and also in refs. 4–17. The principle consists in the visualization of the chromatogram by enzymes that are inhibited by the substances to be analysed and subsequently stained by colour reactions. Thus, the colour develops on the background and colourless spots appear as a consequence of the enzyme-inhibiting action of the substances.

For organophosphorus and carbamate pesticides, cholinesterases have been used as enzymes together with various substrates. Artificial substrates which slowly react with cholinesterase, yielding coloured products either directly or after subsequent reactions, show medium to high sensitivity.

In the present investigation butyrylthiocholine was used as an analogue of the natural substrate in combination with Ellman's reagent together with horse serum cholinesterase. Butyryl- and acetylthiocholine are widely used in the determination of cholinesterase activity[18,19]. This system has high sensitivity and reacts rapidly; both features make it suitable to applications under field conditions where rapid detection of dangerous material is necessary. In case the TLC method is not applicable in such situations, a simplified modification has been designed consisting of a simple strip test.

Paraoxon is used in this study as an example of a cholinesterase inhibitor. The presented TLC–EI is run simultaneously with a conventional TLC and a TLC–EI from the literature using indoxyl acetate as a substrate. The latter has been described to be very sensitive (see refs. 1–3 and 5).

MATERIALS

TLC plates (silica gel 60 F_{254}, 20 × 20 cm × 0.5 mm) were obtained from E. Merck (Darmstadt, G.F.R.). Cholinesterase from horse serum (lyophilised, 4 U/mg) was purchased from E. Merck. Butyrylthiocholine iodide was research grade (Serva, Heidelberg, G.F.R.). Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid), pure] was obtained from Serva and indoxyl acetate, methanol, *n*-hexane, diethyl ether and ethanol (all analytical-reagent grade) from E. Merck. $Na_2B_4O_7$, NaOH, NaH_2PO_4,

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Na_2HPO_4 and NaCl were commercial products. Filter paper circles were of diameter 12.5 cm (Nr. 602 h, Schleicher & Schüll, Dassel, G.F.R.).

METHODS

TLC

Portions (each 5 μl) of 10^{-2}–10^{-8} *M* paraoxon solutions in *n*-hexane were applied to the starting line of the TLC plate. The eluent consisted of *n*-hexane–methanol–diethyl ether (3:1:1) and was allowed to migrate nearly to the top of the TLC plate.

Conventional TLC detection

The paraoxon can be visualized by alkaline hydrolysis when it forms the yellow 4-nitrophenolate anion. With aqueous alkali this reaction occurs very slowly because paraoxon has a poor solubility in water. Therefore 10% methanolic NaOH was used as a spray reagent, which led to rapid decomposition at room temperature.

TLC detection with EI

(a) After evaporation of the eluent the TLC plate was sprayed with a solution of 0.5 mg/ml horse serum cholinesterase in 50 m*M* sodium phosphate buffer (pH 7.7) and dried in the air for about 1 h.

(b) Instead of horse serum cholinesterase a 1:10 diluted human pool plasma in the phosphate buffer was used.

(c) The horse serum cholinesterase was dissolved in 10 m*M* sodium tetraborate buffer (pH 9.2).

Colour development with Ellman's reagent and butyrylthiocholine after TLC–EI

The spray reagent consisted of 1.5 m*M* Ellman's reagent and 7 m*M* butyrylthiocholine in the above mentioned phosphate buffer. Ellman's reagent is stable for several weeks in the buffer if kept cool. Butyrylthiocholine has to be stored as a concentrated solution in distilled water (*e.g.* 70 m*M*) and should be diluted with Ellman's reagent to the final 7 m*M* concentration each day when it is needed. White spots on a yellow background, developed at the very latest 5 min after spraying the plates, show the presence of paraoxon. This spray reagent is used with the TLC–EI (a) and (b) above.

Colour development with indoxyl acetate

The spray reagent was prepared immediately before use by dissolving 15 mg of indoxyl acetate in 1 ml of absolute ethanol and subsequent addition of 10 ml of the previously described borate buffer. This method is a modification of the method used by Mendoza[1–3] and Bogusz and Borkowski[5]. The modification seems to work better under the conditions of the present work. White spots on a blue background, appearing up to 30 min after spraying the plates, show the presence of paraoxon. The described spray reagent is used with the TLC–EI (c) above.

Strip test for paraoxon detection

(a) Filter paper strips were impregnated with 1 mg/ml horse serum cholines-

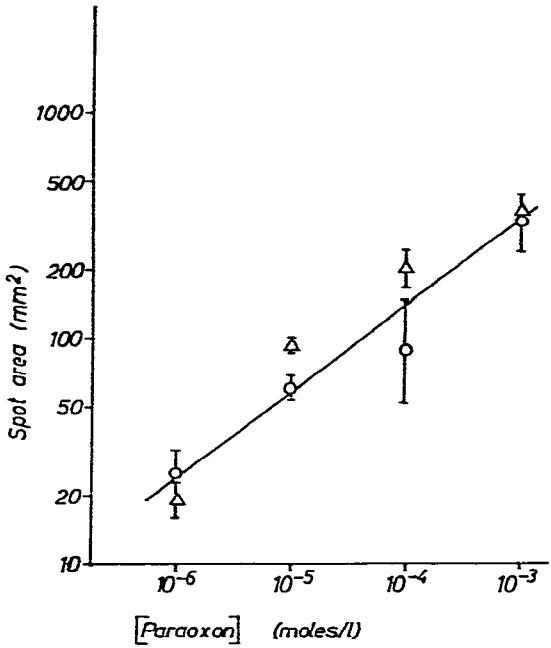

Fig. 1. Double logarithmic plot of spot area *vs.* paraoxon concentration for TLC–EI with indoxyl acetate (△, $n = 3$) and Ellman's reagent (○, $n = 4$). (The area of elliptic spots was calculated after measurement of the long and short axis of the ellipse. Deformed spots at higher paraoxon concentrations were evaluated by counting of squares.)

terase solution in the aforementioned phosphate buffer, dried in the air and kept in a cool dry place. For the test they are slightly wetted with an aqueous paraoxon sample for 5 min. Then they are wetted again with the Ellman's reagent–butyrylthiocholine mixture mentioned above. A blank strip is compared with the test strip, which is treated in the same way with the exception that the aqueous sample did not contain paraoxon nor any other cholinesterase inhibitor. If the blank strip shows a more intense yellow colour than the test strip 5 min after reagent addition, the test is positive.

(b) The filter paper was impregnated with undiluted human pool plasma instead of horse serum cholinesterase. All other procedures are carried out as outlined under (a).

RESULTS

In all cases, TLC showed good reproducibility since the spot magnitude was fairly well correlated with the amount of paraoxon applied (see Fig. 1). The R_F-value was 0.31. The detection limits of the different methods are summarized in Table I.

DISCUSSION

The results clearly show the superiority of the enzymic methods over conventional TLC. Purified horse serum cholinesterase yields (at least for paraoxon) better

sensitivity than human plasma and is claimed to work as well as bovine liver homogenate[5]. Both the new enzymic and the indoxyl acetate reference methods show a high degree of sensitivity. The advantage of the new method is the rapid colour development, which may be useful in emergency cases*. As already mentioned, the time needed for the spot development at the detection limit is *ca.* 5 min for the butyrylthiocholine and 30 min for the indoxyl acetate method. The detection limit is equal for both methods (see Table I). A drawback of the new method as compared to the reference method is the instability of the colour, as the coloured 3-carboxy-4-nitrothiophenolate anion generated during the reaction is slowly oxidized back to Ellman's reagent (see ref. 20). This drawback however is not serious, since the colour does not disappear rapidly and in emergency determinations one would watch the colour development in order to get the information as soon as possible.

TABLE I

DETECTION LIMITS OF DIFFERENT TLC METHODS

Method	*Enzyme*	*Substrate*	*Chromogen*	*Approximate detection limit (ng)*
TLC, no EI (alkaline hydrolysis)			Paraoxon	500
TLC–EI	Human plasma	Butyrylthiocholine	Ellman's reagent	3
TLC–EI	Horse serum cholinesterase	Butyrylthiocholine	Ellman's reagent	0.3
TLC–EI	Horse serum cholinesterase	Indoxyl acetate	Indoxyl acetate	0.3
Strip-test	Human plasma	Butyrylthiocholine	Ellman's reagent	0.5*
Strip-test	Horse serum cholinesterase	Butyrylthiocholine	Ellman's reagent	0.1*

* One drop of sample applied.

The new method, as well as all the other TLC–EI methods, should be suitable to all cholinesterase inhibiting organophosphorus and carbamate compounds. Those organophosphorus compounds that do not inhibit *in vitro* have to be activated by appropriate methods, *e.g.* that described by Ackerman *et al.*[4]. This is necessary in the described strip-test as well, if such compounds occur.

The latter test is a simple but very useful tool for rapidly checking the possible danger from cholinesterase inhibitors directly where they occur. Clearly, with this test a qualitative analysis is impossible, but the cholinesterase inhibiting power of the investigated sample should be correlated to possible dangerous effects in the organism. The new TLC–EI method, on the other hand, is useful for qualitative analysis and is also sufficiently reproducible for semi-quantitative determinations. The latter may be carried out graphically using the regression line shown in Fig. 1.

* In these cases a shorter treatment of the TLC plates with the enzyme, *e.g.* 10 min, is recommended as the loss of sensitivity is small compared with the treatment described under Methods.

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