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Short communication

Toxicological evaluation of harmful substances by in situ enzymatic and biological detection in high-performance thin-layer chromatography

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Abstract

The efficiency of a chromatographic analysis method is determined by the selectivity of the chromatographic separation and the specificity of the detection method. In the case of high-performance thin-layer chromatography (HPTLC) the separated components can be detected and quantified directly on the chromatogram by physical and chemical methods. By coupling high-performance thin-layer chromatography with biological or biochemical inhibition tests it was possible to detect toxicologically active substances in situ. A linear relationship was shown between the signal of the inhibition of cholinesterase and the concentration of the inhibitor using a constant enzyme concentration and a constant incubation time. The graph of the inhibition of the luminescence of *Photobacterium vibrio fisheri* in relation to the concentration of pentachlorophenol (range 20–80 ng) is nearly linear. Measurements were done by using a densitometer or a videodensitometric scanner.

Keywords: Detection, TLC; *Photobacterium* spp; Enzyme inhibitors; Pesticides; Cholinesterase; Paraoxon

1. Introduction

For detection of toxic effects in the environment, the use of biosensors, e.g. inhibition of the growth of microorganism or enzyme-inhibition, is increasingly important. Biomonitoring means that most of the time the results show the summation of an effect in the test system.

Instrumental analytical methods like gas chromatography or liquid chromatography are used for physical or microchemical detection, whereas biomonitoring detects toxicity. Difficulties can arise when unknown toxic substances or metabolites are involved.

In the case of high-performance thin-layer chromatography (HPTLC) the separated components can be detected and quantified directly on the chromatogram by physical (e.g. UV, Vis, Fourier transform-IR, Raman) and chemical methods. There are sufficient microchemical derivatisation methods – e.g. the use of reagents to identify sulfur containing insecticides [1] – and physiological, biochemical and toxicological methods for determining a profile of harmful effects to allow further confirmation.

Cholinesterase, an enzyme which is irreversibly inhibited by harmful substances such as organophosphates, carbamates or some organochlor compounds and metabolites is often used [2]. The quantification of the inhibition can either be estimated by densitometric measurement (TLC scanner) or by using a videodensitometric scanner.

The comparatively quick and low-cost bioassay with the luminescent marine bacterium *Photobac-*

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terium phosphoreum, strain NRRL-B-11177, has gained a considerable popularity for the monitoring of various industrial effluents and for the determination of the toxicity of different chemicals. Many toxic substances (nearly 1350 individual organic compounds) show an inhibition of the bioluminescence of *Photobacterium phosphoreum* and *Vibrio fisheri* in vitro [3]. According to an European patent application of Weisemann et al. in HPTLC these substances have been identified post-chromatographically in situ by dipping the plate into a suspension of bacteria [4] and determining the difference of photon emission using a cooled charged coupled device (CCD) camera.

2. Experimental

2.1. Chemicals

Cholinesterase from bovine serum (EC 3.1.1.8), bovine serum albumin, dichloromethane, Fast Blue salt B, ethanol, ethyl acetate, *n*-hexane, methanol, naphthyl acetate, 2-propanol, tetrahydrofuran and Tris were obtained from Merck (Darmstadt, Germany).

2.2. Apparatus

The following equipment was used: HPTLC chamber for 10×10 cm plates (Desaga, Heidelberg, Germany), Linomat IV (Camag, Muttenz, Switzerland), TLC Scanner CD6O (Desaga, Heidelberg, Germany), TLC-Scanner 3 (Camag, Hüttens, Switzerland), Tauch-fix dipping device (Baron, Isle of Reichenau, Germany), precoated HPTLC silica gel 60 plates F_{254} 10×10 cm (Merck 5365), Sharp Scanner JX-330 with Image Master Software (Pharmacia Biotech, Freiburg, Germany) Peltier Cooled CCD Camera AT1 with software (Dilor, Bensheim, Germany), Ratio Turbidimeter (HACH, Loveland, CO, USA).

2.3. Standard solutions

Working standards were prepared by dissolving the organophosphates, carbamates and pentachlorophenol in methanol.

2.4. Purification of the plates

Before application of the samples the layers were prewashed by developing once with 2-propanol and dried at 110°C for 30 min.

2.5. Application mode

Paroxon was applied to the layer in 5 mm bands. The distance between the bands was 5 mm. The speed of application was 6 s/μl.

2.6. Chromatographic separation

Method:	One-dimensional development performed in a HPTLC chamber without chamber saturation at room temperature
Stationary phase:	Precoated HPTLC silica gel 60 F_{254} (10×10 cm)
Mobile phase:	Tetrahydrofuran–*n*-hexane (7:25, v/v) for the organophosphates and carbamates (10 ml) *n*-hexane–ethyl acetate (6:4, v/v) for the pentachlorophenol (10 ml)
Migration distance:	5 cm
Migration time:	15 min

2.7. Solution of cholinesterase

11 mg cholinesterase (50 U/mg) were dissolved in 180 ml 0.05 *M* Tris–HCl buffer pH 7.8. To stabilize the activity of the enzyme on the plate 0.1% bovine serum albumin was added. This solution could be stored at 4°C for 3 weeks. The activity of the enzyme was controlled before the tests [5].

2.8. Solutions of the substrates

Solution 1:	250 mg 1-naphthyl acetate were dissolved in 100 ml absolute ethanol.
Solution 2:	400 mg Fast Blue salt B were

Table 1
Composition of the cultivation broth

Substance	Concentration
Sodium chloride; NaCl	30 g/l
Disodium hydrogenphosphate, $Na_2HPO_4 \cdot 2H_2O$	4.65 g/l
Potassium dihydrogenphosphate KH_2PO_4	1.0 g/l
Magnesium sulfate $MgSO_4 \cdot 7H_2O$	0.1 g/l
Glycerine	3 ml/l
Peptone from casein	5 g/l
Yeast extract	2 g/l

The pH was adjusted to 7.2±0.2 by HCl or NaOH.

dissolved in 160 ml distilled water.

Dipping solution: 1 part of solution 1 and 4 parts of solution 2 were mixed just before use.

2.9. *Cultivation of the bacteria strain*

Photobacterium fischeri, strain NRRL B-11177, was cultivated in cultivation broth for 17±1 h at 20±0.2°C in Erlenmeyer flasks shaking with 180 U/min (see Table 1).

After incubation, the density of the bacterial suspension was estimated with a turbidimeter. As a standard for calibration a solution of formazin was used according to the German standard DIN 38 404 part 2, Chapter 3.2.2–3.2.3. The results were presented as units of formazin (FAU).

As dipping solution, a suspension of bacteria in cultivation broth was used with a density of 20–30 FAU.

3. Procedure

After the separation, the chromatograms were dried in a stream of warm air for 3 min and then immersed in the various dipping solutions. Oxidation of the organophosphorus compounds was carried out with bromine vapour as described elsewhere [6].

3.1. *Enzyme inhibition*

The HPTLC plate was dipped into the solution of cholinesterase (2 s), re-dried and incubated for 30 min at 37°C in an incubation chamber with 90% humidity. The chromatogram was then immersed for 2 s in the dipping solution of substrates. After 3 min white inhibition spots could be detected on a violet background (see Fig. 1). The in situ quantitation was performed either by absorption photometric analysis in the reflectance mode at λ=533 nm (Fig. 2) or by detecting the differences of colour intensities by a videodensitometric scanner (Fig. 3).

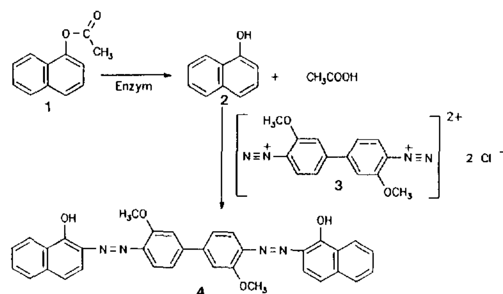

Fig. 1. Enzymatic reaction of cholinesterase on a TLC plate: (1) Naphthyl acetate; (2) naphthol; (3) Fast Blue salt B; (4) diazonium dye.

3.2. *Inhibition of the bioluminescence of Photobacterium phosphoreum* [4]

The HPTLC plate was dipped for 2 s into the dipping solution of a suspension of bacteria (20 FAU), re-dried and covered with a plate of glass. Toxic substances could be identified post-chromatographically in situ on the chromatogram by detecting

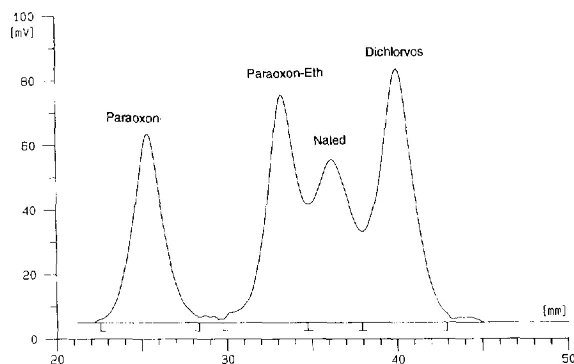

Fig. 2. Chromatogram of organophosphorus insecticides; detection mode: cholinesterase inhibition on a TLC plate, paraoxon-ethyl (0.4 ng), naled (0.4 ng), dichlorvos (2 ng); TlC-scanner absorbance in reflectance mode at λ=533 nm.

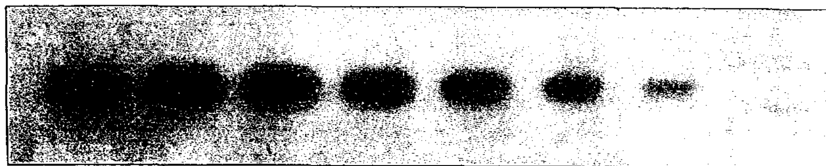

Fig. 3. Video scan report of cholinesterase inhibition on a TLC plate by oxamyl in the range of 0.8–32 ng/spot.

2–3 min the differences of photon emission using a cooled CCD camera in a dark chamber.

4. Results and discussion

A linear relationship between the signal of the inhibition of cholinesterase and the concentration of the inhibitor (paraoxon between 20–400 pg/5 mm band) could be shown using a constant enzyme concentration and a constant incubation time.

$$y = 1.124x + 7.01$$

$$[r = 0.99957;\ \text{S.D.} = 5.265;\ n = 7]$$

At higher concentrations of the inhibitor the results showed a calibration curve with second order polynomial regression. Table 2 shows the detection limits of several organophosphates, carbamates and pentachlorophenol. The detection limit should be proportional to the inhibition constant of the particular substance and can lie in the lower picogram range. The determination of the inhibition constant itself was performed in vitro. In vitro tests can only find the summation of the inhibitory effect. But the biochemical detection in HPTLC could show that several working standards, such as parathionethyl, butocarboxim and aldicarb are very often contaminated with more toxic impurities, which can only be detected after chromatographic separation [6].

A linear correlation between the inhibition of the bioluminescence of *Photobacterium phosphoreum* and the concentration of pentachlorophenol could be shown.

$$y = 50.13x + 0.305$$

$$[r = 0.99615;\ \text{S.D.} = 0.6269;\ n = 6]$$

The graph of the inhibition of the luminescence of *Photobacterium vibrio fisheri* in relation to the concentration of pentachlorophenol range 20–80 ng) is almost linear. Under the conditions described above the detection limit of pentachlorophenol was found to be between 10 and 20 ng.

Using biological and biochemical detection methods, the presence of toxic metabolites could be shown by photosensitive destruction of organochlorines in situ.

After detection of the separated components by HPTLC and direct quantification on the chromato-

Table 2
Detection limit of inhibitors of cholinesterase in HPTLC

Substance	Detection limit (ng)	Inhibition constant k_i (l mol^{-1} min^{-1}) [7]
Parathion-ethyl,after oxidation	0.045	–
Paraoxon-ethyl	0.013	$4.9 \cdot 10^5$
Paraoxon-methyl	0.400	$2.2 \cdot 10^4$
Mevinphos	0.200	$1.4 \cdot 10^4$
Dichlorvos	0.200	$5.2 \cdot 10^4$
Carbaryl	0.200	$2.7 \cdot 10^4$
Aldicarb	0.400	$1.6 \cdot 10^4$
Butoxycarboxim	0.100	$3.2 \cdot 10^3$
Butocarboxim	0.800	$1.6 \cdot 10^3$
Oxamyl	0.800	$1.4 \cdot 10^5$
Pentachlorophenol	20.000	$1.0 \cdot 10$

gram by physical methods, it was possible to use the same chromatogram in biological or biochemical inhibition tests to evaluate toxicologically active substances in situ. A linear relationship between the signal of the inhibition effect and the concentration of the inhibitor was shown.

Using this method it should be possible to look for harmful substances in the environment or to prove previous exposure of people. This method can contribute a lot of information about unknown toxins and unknown metabolites, for example during photocatalysed degradation of waste water. In combination with biosensors this method is suitable as a valid confirmatory test.

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