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## Note

# Determination of cholinesterase-inhibiting pesticides and some of their metabolites in cases of animal poisoning using thin-layer chromatography

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In veterinary practice one is regularly confronted with incidents involving unwanted side-effects, abuse or —more frequently— misuse of agrochemicals. Domestic animals and wildlife, especially birds, may be affected. Annually 100–250 cases of suspected acute pesticide intoxication in animals need to be diagnosed, the majority of them being caused by cholinesterase-inhibiting compounds. Hence a rapid screening method for representatives of this group in gizzard and gastrointestinal contents as well as in baits, food and environmental samples was necessary.

A thin-layer chromatographic (TLC) method for qualitative and semiquantitative analysis of some hundred pesticides and metabolites was developed, most of them belonging to the class of organophosphates. Although less relevant in veterinary practice from a diagnostic point of view, the present method includes (thio-)carbamates, carbamoyloximes, dithiocarbamates and ureas too. These compounds exhibit cholinesterase-inhibiting properties *in vitro*, by which they may interfere during analysis.

### *Principle of the method*

Samples were ground with anhydrous sodium sulphate and extracted with dichloromethane. In general, the pesticides extracted were subjected to TLC without additional cleaning. The extracts were spotted on silica gel HPTLC plates and developed with either xylene, di-*n*-butyl ether, *n*-butyl acetate or methyl isobutyl ketone. After evaporation of the solvent and, if necessary, an activation of the spots by bromine vapour, the plates were sprayed and incubated with bovine liver suspension. To detect cholinesterase inhibition, the plates were sprayed with the substrate 2-naphthyl-acetate and Fast Blue B salt as the chromogenic agent.

## EXPERIMENTAL

### *Chemicals*

All chemicals were of analytical grade from E. Merck (Darmstadt, F.R.G.) unless specified otherwise.

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*Pesticide standard solutions*

Pesticide reference standards of >99% purity were obtained from the U.S. Environmental Protection Agency, and from Lamers & Pleuger ('s-Hertogenbosch, The Netherlands). For TLC, standard solutions were prepared in dichloromethane (residue analytical quality). Both reference standards and standard solutions were stored at 4°C.

*HPTLC plates*

Ready to use HPTLC glass plates 20 cm × 10 cm, coated with silica gel 60 $F_{254}$ (Merck), were employed.

*Preparation of enzyme solution*

A 20-g amount of fresh beef liver was homogenized in a Sorvall Omni Mixer with 200 ml of 0.05 *M* Tris–HCl buffer pH 8.2 containing 0.1% (v/v) Triton X-100 followed by centrifugation for 5 min at 150 *g*. Aliquots of 4 ml of the supernatant were transferred to plastic tubes and freezed at –20°C. Prior to use, portions were each diluted in 12 ml of distilled water. One portion suffices for the spraying of two 20 cm × 10 cm plates.

*Preparation of substrate solution*

Solution A: 1.25 g/l 2-naphthyl acetate (biochemical grade) in ethanol. Solution B: 1.56 g/l Fast Blue B (for microscopy) in water. Immediately before use, 4 ml of solution A were mixed with 16 ml of solution B. This suffices for the spraying of four 20 cm × 10 cm plates.

*Sample extraction*

Amounts of 5–10 g of gizzard or gastrointestinal content, bait, food or environmental sample were ground in a mortar with anhydrous sodium sulphate (residue analytical quality) and sea-sand to a free flowing powder. The powder was extracted with 10–25 ml of dichloromethane (residue analytical quality) in a conical flask by mechanical shaking for 1 h, followed by filtration through Whatman No. 41 paper.

*TLC*

Aliquots of extracts and different concentrations of pesticide standard solutions were spotted on the HPTLC plates using micropipettes (5 × 1 μl). The plates were developed in either xylene, di-*n*-butyl ether (spectroscopic grade), *n*-butyl acetate or methyl isobutyl ketone in an unsaturated chamber to a distance of about 6 cm from the origin. After evaporation of the solvents, the plates were exposed to bromine vapour for 30 s and kept in a ventilated hood for 10 min in order to evaporate the excess of bromine. The plates were then uniformly sprayed with 6 ml of enzyme solution and placed for 30 min in an incubator with 80–85% relative humidity at 37°C. Next, the plates were sprayed with freshly prepared substrate solution and left at room temperatue. White spots appeared on a magenta background after some minutes and were marked with a pencil. Subsequently, $hR_F$ values were calculated.

## RESULTS AND DISCUSSION

The present method is a modification of that by Ackermann[1–4]. Dichloromethane was preferred to chloroform as the extraction solvent: due to its higher volatility, it evaporates faster during spotting, resulting in smaller spot areas at the origin, thus enhancing resolution and sensitivity. For all the pesticides tested, the solubility in dichloromethane was $\geqslant 1$ mg/ml. Although chloroform might be a more efficient extraction solvent for some pesticides with high polarity, dichloromethane suffices for the present application of acute intoxications with high levels of pesticides. No significant differences were observed between chloroform or dichloromethane extracts as judged from interfering spots resulting from matrix components. Moreover, dichloromethane is less toxic.

The enzyme solution needed a Tris–HCl buffer of pH 8.2. Unbuffered homogenization resulted in severe loss of activity. Furthermore, Triton X-100 was added in order to achieve a better disposition of the esterases involved, resulting in an increased activity.

Table I shows the mean $hR_F$ values of some hundred cholinesterase inhibiting

TABLE I

$hR_F$ VALUES OF CHOLINESTERASE-INHIBITING PESTICIDES AFTER HPTLC ANALYSIS

Values are means from at least three experiments.

| *Compound* | $hR_F$ | | | |
|---|---|---|---|---|
| | *Solvent* | | | |
| | *Xylene* | *Dibutyl ether* | *Butyl acetate* | *Methyl isobutyl ketone* |
| Acephate | 5 | 35 | 95 | 98 |
| Aldicarb | 0 | 2 | 47 | 74 |
| Aldicarbsulfone | 0 | 0 | 8 | 30 |
| Aldicarbsulfoxide | 0 | 0 | 0 | 0 |
| Asulam | 0 | 0 | 41 | 75 |
| Azamethiphos | 0 | 0 | 42 | 59 |
| Azinphosmethyl | 3 | 17 | 81 | 91 |
| Bendiocarb | 0 | 13 | 79 | 92 |
| Benomyl | 0 | 20 | 89 | ns* |
| Bromophos | 82 | 92 | 96 | 97 |
| Bromophosethyl | 86 | 96 | 97 | 98 |
| Butocarboxim | 0 | 0 | 43 | 70 |
| Butoxycarboxim | 0 | 0 | 7 | 27 |
| Butylate | 15 | 80 | 97 | 98 |
| Carbaryl | 2 | 18 | 80 | 92 |
| Carbetamide | 0 | 1 | 30 | 67 |
| Carbophenothion | 78 | 96 | 98 | 98 |
| Carbofuran | 0 | 5 | 70 | 87 |
| Chlorbromuron | 4 | 18 | 75 | 90 |
| Chlorbufam | 32 | 87 | 97 | 98 |
| Chlorphenvinphos | 0 | 7 | 70 | 81 |

TABLE I (*continued*)

| *Compound* | $hR_F$ | | | |
|---|---|---|---|---|
| | *Solvent* | | | |
| | *Xylene* | *Dibutyl ether* | *Butyl acetate* | *Methyl isobutyl ketone* |
| Chlorpropham | 29 | 81 | 95 | ns* |
| Chlorpyriphos | 81 | 95 | 98 | 98 |
| Chlorpyriphoxon | 0 | 10 | 74 | 90 |
| Chloroxuron | 0 | 0 | 32 | 67 |
| Chlortoluron | 0 | 0 | 39 | 73 |
| Coumaphos | 7 | 54 | 92 | 95 |
| Coumaphoxon | 0 | 0 | 33 | 69 |
| Cycloate | 9 | 67 | 94 | 98 |
| Demeton-S-methyl | 0 | 0 | 30 | 64 |
| Demeton-S-methylsulfon | 0 | 0 | 6 | 22 |
| Diallate | 44 | 91 | 98 | 98 |
| Diazinon | 3 | 40 | 88 | 92 |
| Diazoxon | 0 | 0 | 6 | 12 |
| Dibrom | 0 | 11 | 67 | 82 |
| Dichlofenthion | 82 | 94 | 97 | 97 |
| Dichlorvos | 0 | 5 | 48 | 68 |
| Difenoxuron | 0 | 0 | 28 | 64 |
| Diflubenzuron | 4 | 32 | 89 | ns* |
| Dimethoate | 0 | 0 | 17 | 46 |
| Diuron | 0 | 0 | 43 | 73 |
| Eptam | 12 | 74 | 91 | 98 |
| Ethiophencarb | 0 | 15 | 80 | 92 |
| Ethephon | 0 | 0 | 0 | 0 |
| Ethoprophos | 0 | 10 | 68 | 87 |
| Etrimfos | 9 | 67 | 98 | 98 |
| Fenitrothion | 43 | 72 | 93 | 96 |
| Fenoxycarb | 2 | 22 | 84 | 94 |
| Fenthion | 51 | 82 | 94 | 97 |
| Fonofos | 54 | 89 | 96 | 97 |
| Fonofoxon | 0 | 3 | 51 | 56 |
| Formothion | 3 | 8 | 82 | 92 |
| Glyphosate | 0 | 0 | 0 | 0 |
| Heptenofos | 0 | 0 | 47 | 68 |
| Isofenphos | 9 | 75 | 97 | 97 |
| Isoproturon | 0 | 2 | 34 | 66 |
| Jodfenphos | 80 | 91 | 97 | 98 |
| Linuron | 4 | 14 | 75 | 93 |
| Malaoxon | 0 | 0 | 48 | 73 |
| Malathion | 5 | 37 | 92 | 96 |
| Methabenzthiazuron | 0 | 4 | 43 | 71 |
| Methamidophos | 0 | 0 | 5 | 12 |
| Methidathion | 10 | 39 | 92 | 96 |
| Methiocarb | 3 | 20 | 79 | 92 |
| Methomyl | 0 | 0 | 22 | 57 |
| Metobromuron | 3 | 15 | 7 | 89 |
| Metoxuron | 0 | 0 | 27 | 59 |

(*Continued on p. 452*)

TABLE I (*continued*)

| *Compound* | $hR_F$ | | | |
|---|---|---|---|---|
| | *Solvent* | | | |
| | *Xylene* | *Dibutyl ether* | *Butyl acetate* | *Methyl isobutyl ketone* |
| Mevinphos | 0 | 0 | 18–32–40** | 37–55–62** |
| Mevinphos-*cis*-isomer | 0 | 0 | 18–32** | 37–55** |
| Monolinuron | 3 | 15 | 74 | 91 |
| Omethoate | 0 | 0 | 0 | 4 |
| Oxamyl | 0 | 0 | 4 | 22 |
| Oxydemetonmethyl | 0 | 0 | 0 | 0 |
| Paraoxon | 0 | 2 | 48 | 77 |
| Paraoxonmethyl | 0 | 0 | 34 | 62 |
| Parathion | 47 | 83 | 96 | 96 |
| Parathionmethyl | 39 | 71 | 94 | 97 |
| Pencycuron | 2 | 24 | 97 | 97 |
| Phosalone | 21 | 61 | 93 | 97 |
| Phosphamidon | 0 | 0 | 10–32** | 36–65** |
| Phosmet | 7 | 26 | 87 | 95 |
| Phoxim | 52 | 85 | 96 | 98 |
| Pirimicarb | 0 | 1 | 36 | 69 |
| Pirimiphosmethyl | 16 | 75 | 97 | 98 |
| Propetamphos | 7 | 66 | 96 | 98 |
| Propoxur | 0 | 10 | 77 | 93 |
| Pyrazophos | 1 | 28 | 90 | 94 |
| Sodam | 0 | 0 | 0 | 0 |
| Sulfotep | 41 | 86 | 96 | 96 |
| Temephos | 30 | 71 | 96 | 97 |
| Terbufos | 60 | 97 | 98 | 98 |
| Tetrachlorvinphos | 2 | 11 | 78 | 87 |
| Thiofanox | 0 | 3 | 59 | 82 |
| Thiometon | 47 | 83 | 94 | 96 |
| Thiram | 4 | 19 | 83 | 93 |
| Tolclofosmethyl | 69 | 87 | 95 | 96 |
| Triallate | 51 | 91 | 98 | 98 |
| Triazophos | 0 | 17 | 87 | 95 |
| Trichlorphon | 0 | 0 | 13 | 32 |
| Trichloronate | 84 | 94 | 97 | 97 |
| Vamidothion | 0 | 0 | 2 | 9 |

* No spots visible.
** Multiple spots.

compounds. The means are based on at least three observations. In contrast to other reports[1–8], in which low-boiling solvent mixtures were employed, $hR_F$ values of excellent long-term reproducibility were obtained using single solvent systems with relatively high vapour pressures. Deviations of the mean $hR_F$ values were often not more than 3 units at the most. Consequently, preliminary identification of spots proceeded more quickly and with greater confidence. Further decrease of the analysis time was

achieved by employing HPTLC plates in stead of normal TLC plates. Since HPTLC plates have an higher number of plates per unit length, shorter development times were feasible. A skilled analyst is able to perform 20 or more analyses per day.

Identification of inhibiting spots of extracts was mainly based on comparison of $hR_F$ values with those of reference compounds with and without derivatization with bromine vapour prior to development of the plates. Bromine vapour oxidizes the P=S bond, present in most cholinesterase-inhibiting pesticides, to a P=O bond. If for example paraoxon, the oxidized derivative of parathion, is detected on a plate exposed to bromine vapour prior to development, additional evidence is obtained for an intoxication by parathion. Treatment of plates with bromine after development results in a better detection limit of most pesticides, because oxidized organophosphorus compounds are stronger cholinesterase inhibitors than their sulphur analogues. Limits of detection (per spot) are in the range of 0.2–20 ng, with the exception of dimethoate (200 ng), ethephon (2000 ng), ethoprophos (2000 ng), glyphosate (2000 ng), pirimicarb (100 ng) and sulfotep (200 ng). In intoxications with juridical aspects or doubtful TLC results, confirmatory evidence was obtained using gas or liquid chromatography.

In conclusion, the present method provides efficient analysis of cholinesterase-inhibiting pesticides. It was demonstrated to be a valuable diagnostic tool in veterinary toxicology.

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