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Behaviour studies of the fungicide cymoxanil in two strains of the fungus *Botrytis cinerea* and in haemolymph of locust and lobster

I. *In situ* monitoring by internal surface reversed-phase high-performance liquid chromatography

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ABSTRACT

A method for following the metabolism of the fungicide cymoxanil in various biological media is described. By using a recently developed high-performance liquid chromatographic method, with an internal surface reversed-phase column, it is unnecessary to clean up the sample before analysis. Thus this technique makes monitoring in fungi as well as in arthropod haemolymph easier and faster.

INTRODUCTION

Cymoxanil, 1-(2-cyano-2-methoxyiminoacetyl)-3-ethyl urea, is an agricultural fungicide effective against grape downy mildew, which has previously been studied in plants and animals by using radiolabelled material [1–3]. In *Bortytis cinerea*, it was found that a sensitive strain L was able to decompose the fungicide almost completely within 6 h, and that a tolerant strain N metabolized only 20% of cymoxanil [3]. A profungicide behaviour, implying an activation step under hydrolase(s) and leading to fungitoxic metabolite(s) such as M_1 (and/or M_2), and finally to glycine, has been proposed (Fig. 1, path A).

By screening natural or synthesized compounds [4–7] that exhibit various pesticidal acitivities, their behaviour was studied *in vivo* or *in vitro* directly in various biological fluids, such as insect or arthropod haemolymph, without pretreatment. Analytical techniques such as high-field proton nuclear magnetic resonance (^{1}H NMR) [5,6], fast-atom bombardment mass spectrometry (FAB-MS) [7] and in-

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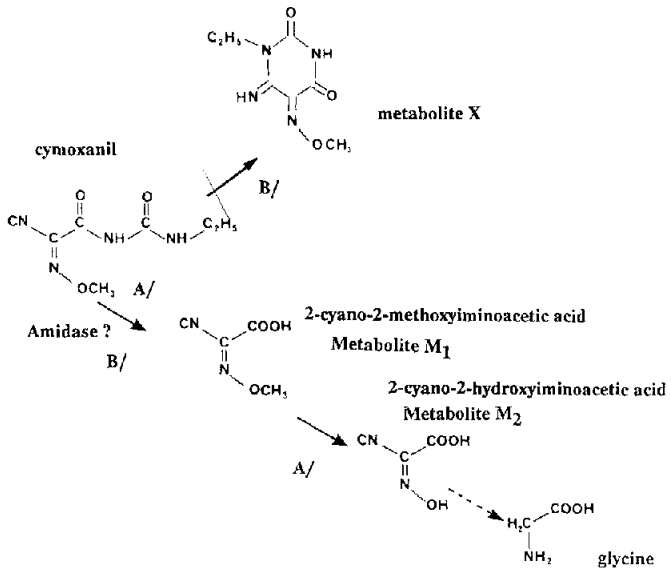


Fig. 1. Metabolism of cymoxanil: (A) in plants and animals [1]; (B) in arthropod haemolymph.

ternal surface reversed-phase (ISRP) high-performance liquid chromatography (HPLC) were used to monitor the xenobiotics.

ISRP-HPLC was initially designed for the direct analysis of various drugs and their metabolites in protein matrices, such as human plasma [9–11], and has recently been applied to plant sample analysis [12]. The combined use of size-exclusion chromatography with reversed-phase separation overcomes the denaturation of proteins observed with conventional reversed-phase columns. The large molecules are eluted in the void volume while the small analytes penetrate the pores and are separated by interacting with the reversed-phase packing.

This method was previously developed for the direct *in vitro* analysis of insecticides or proinsecticides, in insect or lobster haemolymph [8]. Natural toxins, destruxins, have also been monitored in the same way in locust haemolymph, *in vitro* [7] and *in vivo* [14].

The aim of this work was to study a convenient and quantitative method of the behaviour of non-radiolabelled cymoxanil in *B. cinerea* in order to confirm the previous results based on thin-layer chromatography (TLC) and radiolabelled cymoxanil. As a comparison, the metabolism of this xenobiotic was also followed in other biological fluids likely to contain peptidases. Insect and crustacean haemolymphs were chosen for a subsequent study of structures for potential new proinsecticides, as well as the effects of possible pollution (in fresh or saline water) on the metabolic activities of crustacean species, such as crayfish, shrimp, etc.

Given our previous experiments using biological fluids [5–8], the locust, *Locus-*

ta migratoria, and the lobster, *Homarus americanus*, were selected as models in these preliminary studies, in view of the importance of their haemolymph content.

EXPERIMENTAL

Biological samples

Two strains of *B. cinerea* were used in this investigation: the sensitive strain L and the tolerant strain N, as previously described by Leroux *et al.* [3] (L = B and N = VI).

The *L. Migratoria* locusts (male adults) were grown in the Laboratoire d'Evolution des Etres Organisés, Université P. et M. Curie (Paris, France) and the *H. americanus* lobsters were obtained from the Laboratoire de Physiologie de la Reproduction, Equipe de Neuroendocrinologie des crustacés, Université P. et M. Curie.

Columns

The HPLC columns, a Regis Pinkerton GFF 5-μm (50 mm × 4.6 mm I.D.) analytical column and a Regis Pinkerton GFF 5-μm (10 mm × 3 mm I.D.) guard column, were both from Regis and supplied by Touzart et Matignon (Paris, France). HA- and FH-type filters were supplied by Millipore (Molsheim, France). Organic solvents (chromatographic grade; SDS, Vitry sur Seine, France) were filtered through an FH filter (p.5 μm); 18-MΩ deionized water (Water Milli Q apparatus) was filtered through HA filters (0.45 μm).

Structural studies

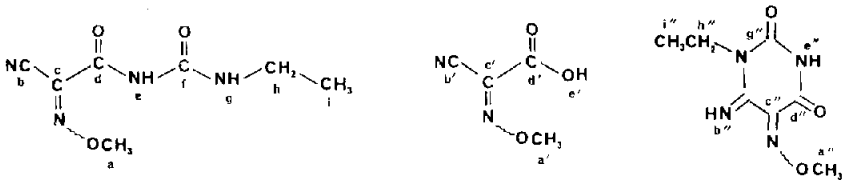
IR, NMR and mass spectra were obtained for cymoxanil and several metabolites (Fig. 2). The following instruments and abbreviations were used. IR Pye Unicam Model SP3-200 [ν cm^{-1} (abbreviations: S = strong; W = weak; s = sharp; b = broad; attribution)].

Bruker Model WM 250 and Bruker Model AM 500 for the ^{1}H NMR and ^{13}C NMR spectra [chemical shift δ in $C^{2}HCl_3$ with respect to TMS as internal reference (multiplicity: s = singlet; t = triplet; q = quadruplet; attribution)].

Quadrupole mass spectrometer Nermag R10 10-C, under chemical ionization (CI) conditions using ammonia as reactant gas [m/z (relative abundance)].

Reagents and chemicals

Pure (99%) and technical (95%) cymoxanil [1-(-2-cyano-2-methoxyiminoacetyl)-3-ethyl urea] were supplied by DuPont De Nemours (Wilmington, DE, U.S.A.). IR (KBr): 3330 (s, –NH–), 3120 (b, –NH–), 3050 (b, –NH–), 2980–2960–2880 (–CH_2 –CH_3, –OCH_3), 2220 (W, –CN), 1720–1700–1680 (S, –C=O). ^{1}H NMR ($C^{2}HCl_3$), δ:8.4 (1H, bs, –N*H*CO–), 7.9 (1H, bs, –N*H*CO–), 4.32 (3H, s, CH_3O–), 3.37 (2H, q, –CH_2 CH_3), 1.22 (3H, t, CH_3–CH_2). In aqueous solution,



	Cymoxanil	M_1 metabolite	X metabolite
MS-CI /NH4+ [MH]$^+$	199 (15)		199 (100)
[MH+NH_4]$^+$	216 (100)	146 (100)	216 (10)
m/z (abundance)			
IR(KBr)	3330, 3120	3400 - 2500	3330, 3200 3080
ν (cm^{-1})	3150, 2980, 2210	2220	
	1720, 1700	1730	1720, 1700
	1680		1600
^{1}H NMR			
($CDCl_3$)	a 4.32	a' 4.36	a" 4.4
	e,g 8.4 and 7.9	e' 12.1	e", g" 10.4 and 8.2
	h 3.37	-	h" 4.0
	i 1.22	-	i" 1.25
^{13}C NMR	a 66.6	a' 66.6	a" 67.2
($CDCl_3$)	b 106.4	b' 107.3	b", c", d" and f':
	c 126.5	c' 125	133.4, 147.2,
	d, f: 151.1, 157.8	d' 160	148.5 and 156.4
	h 35.2	-	h" 37.4
	i 14.8	-	i" 12.5

Fig. 2. Structures of cymoxanil and of the metabolites M_1 and X [15].

the signals are shifted upfield, and the methoxyl signal appears at 4.14 ppm [15]. ^{13}C NMR (C^2HCl_3), δ:157.8 (s, –NH*C*O–), 151.1 (s, –NH*C*O–), 126.5 (s, –*C*–N–), 106.4 (s, *C*N), 66.6 (s, CH_3O), 35.2 (q, CH_2–CH_3), 14,8 (t, CH_3–CH_2–). NH_4^+ CI-MS: [MH]$^+$ 199 (15), [MNH_4]$^+$ 216 (100).

The metabolite M_1 of cymoxanil, 2-cyano-2-methoxyiminoacetic acid, was synthesized (Fig. 3) from ethyl 2-cyano-2-hydroxyiminoacetate (Aldrich, Strasbourg, France) according to ref. 1; m.p. (Buchi) was 51–52°C against 45–47°C in ref. 1 (instrument not specified). IR (KBr): 3400–2500 (b, –OH), 2220 (W, –CN), 1730 (S, –C=O). ^{1}H NMR (C^2HCl_3), δ:12.6 (1H, s, COO*H*), 4.36 [3H, s, CH_3O–

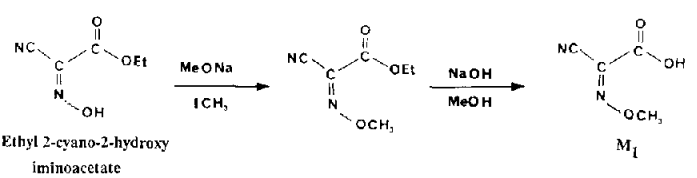


Fig. 3. Synthesis of the metabolite M_1 [1].

(4.01 in aqueous solution)]. ^{13}C NMR (C^2HCl_3), δ:160.0 (s, –*C*OOH), 125.0 (s, –*C*=N–), 107.3 (s, –*C*N), 66.6 (s, *C*H_3O–). NH_4^+ CI-MS: $[M+N_2H_7]^+$ 163 (20), $[MNH_4]^+$ 146 (100), $[MH-H_2O]^+$ 110 (10).

The metabolite X, which is more polar than cymoxanil, was obtained by extraction from a preliminary incubation of lobster haemolymph with cymoxanil.

Preparation of B. cinerea samples (Fig. 4)

The two strains of *B. cinerea* were maintained on potato dextrose agar (PDA). After fifteen days, mycelial pellets of *B. cinerea* were prepared by seeding $7 \cdot 10^5$ conidia per ml in a yeast extract liquid medium [16] and incubated at 23°C on a rotary shaker at 150 rpm for 24 h. Then, 3 ml of these cultures were inoculated with 150 ml of yeast extract medium in 300-ml Erlenmeyer flasks and incubated again for 24 h as described above to obtain the mycelium used in the experiments. This 48-h mycelium was harvested by filtration through a gauze (250 μm) and resuspended in an equal volume of yeast extract medium before treatment with 20 μg/ml cymoxanil ethanol solution. The final concentration of ethanol was the same in control and treated cultures and never exceeded 0.5%. After 0, 2 and 6 h, the mycelium (My) was separated from the culture medium (CM). Each fraction (My and CM) was frozen, lyophilized (2.5 g for CM and 0.4 for My), ground and stored at −30°C until analysis. Then aliquots (75 mg in the case of CM) were diluted in 1 ml of water–tetrahydrofuran (THF) (95:5, v/v) in order to provide a maximum cynoxanil concentration of *ca.* $6 \cdot 10^{-4}$ *M* for the T_0 time samples (assuming that cymoxanil remains entirely in the CM fraction). The samples were filtered through a 0.5-μm HA membrane before HPLC injection.

The lobster haemolymph was extracted from the pericardial cavity level of a male *H. americanus* lobster [8] and diluted (1:1, v/v) with a 10% solution of trisodium citrate in order to minimize protein coagulation. After elimination of the haemocytes by centrifugation (1000 *g*, 4°C) the haemolymph sample was stored at −60°C until analysis. In a typical experiment, a sample of 500 μl of

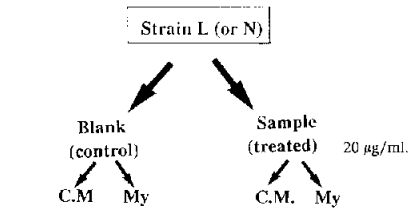


Fig. 4. Preparation of various samples of the fungus *B. cinerea*.

haemolymph was incubated at time T_0 with 500 μl of $1.4 \cdot 1^{-3}$ *M* cymoxanil water–THF (95:5, v/v)] solution. Aliquots of 5 μl of the resulting mixture were injected after centrigufation.

The locust haemolymph was sampled from male locusts (*L. migratoria*) as decribed elsewhere [5,14], and was immediately incubated with an equal volume of $1.4 \cdot 10^{-3}$ *M* cymoxanil water–THF (95:5, v/v) solution.

Extraction and structure determination of metabolite X

Lobster haemolymph solution (10 ml) was incubated for 5 h with 10 ml of a $2 \cdot 10^{-2}$ *M* cymoxanil water–THF (95:5, v/v) solution. The mixture was diluted with water and extracted twice with 20 ml of diethyl ether. After concentration, the extracts were chromatographed [preparative TLC 20 × 20 cm, silica Merck 60 PF_{254}, 1 mm thickness; eluent chloroform–methanol (95:5, v/v)] (Fig. 5). Owing to the retention order in reversed-phase chromatography (M_1, X, cymoxanil), compound X was expected to elute after the cymoxanil band ($R_F = 0.72$). In fact, extraction of the next band ($R_F = 0.43$) gave 2.0 mg of a crystalline compound (m.p. 214°C) with the same capacity factor k' as the metabolite X.

IR (KBr): 3330 (s and S, –NH–), 3200 (b, NH), 3080 (b, NH–), 1720-1710 (S, C–O), 1600 (S). ^{1}H NMR (C^2HCl_3), δ:10.4 (1H, bs, –N*H*–), 8.2 (1H, bs, –N*H*–), 4.42 (3H, s, C*H*$_3$O–), shifted upfield to 4.16 ppm in aqueous solution [15]), 4.09 (2H, q, CH_3CH_2–), 1.25 (3H, t, C*H*$_3$–CH_2–). ^{13}C NMR (C^2HCl_3), δ:158.3, 148.5, 147.5 and 133.1 (4s, –*C*O– and –*C*=N–), 67.2 (s, –CH_3O), 37.4 (q, CH_3CH_2–), 12.5 (t, *C*H$_3$–CH_2), NH_4^+ CI-MS: $[MNH_4]^+$ 216 (10), $[MH]^+$ 199 (100), $[M + NH_4 - HCN]^+$ 187 (12), $[MH - HCN]^+$ 172 (8).

Identification of metabolite M_1 in lobster haemolymph

This was performed by overloading the biological medium during the reaction

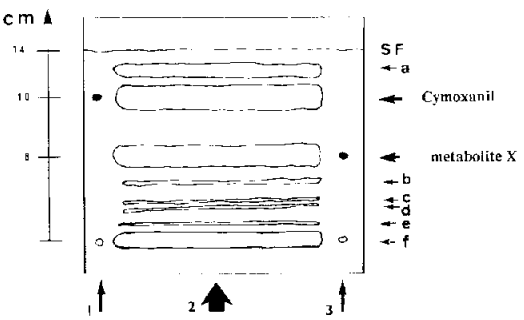


Fig. 5. Preparative TLC separation of metabolite X after incubation of lobster haemolymph by cymoxanil. TLC conditions: plates 20 × 20 cm silica Merck 60 PF_{254}, 1 mm thickness; eluent, methanol–chloroform (5:95, v/v); UV detection at 254 nm. Lanes: 1 = cymoxanil as reference; 2 = extract from lobster haemolymph; 3 = metabolite X as reference. SF = Solvent front; R_F for cymoxanil = 0.72; R_F for metabolite X = 0.43; a–e, secondary bands (not studied); f, deposit due to polar components of the extract.

with an authentic sample of M_1 in the HPLC experiments ($k' = 0.85$), as well as by ^1H NMR (appearance of a signal at 4.01 ppm [15]).

Chromatographic instrumentation

The Beckman HPLC system consisted of Model 112 pump with a Model 421 monitor, and an Altex injection valve equipped with a 5-μl loop. The UV detector was a Model 165 set at 254 or 230 nm and at 0.5 a.u.f.s.

Calibration curves

Standards were prepared by adding known amounts of cymoxanil to untreated samples of strain L or N, in order to provide a concentration range of 0.25–$10.08 \cdot 10^{-4}$ *M* in the L strain and $0.17–12.34 \cdot 10^{-4}$ *M* in the N strain. Calibration curves obtained by plotting the cymoxanil peak height against its concentration were linear within the ranges measured. Cymoxanil was quantified in the biological medium by measuring the peak height and using the calibration curves.

RESULTS AND DISCUSSION

Behaviour of cymoxanil in B. cinerea strains

The disappearance of cymoxanil in *B. cinerea* was monitored by the decrease of the corresponding chromatographic peak. Adjustment of the eluent led to a capacity factor of 1.3 for this compound by using 3% of the organic modifier THF–2-propanol (6:10, v/v) in phosphate buffer (0.1 *M*, pH 7.0). Under these conditions, after simple filtration through a membrane, aliquots of the various samples (Fig. 4) of the *B. cinerea* strains L and N were directly injected into ISRP-HPLC columns.

Owing to the great heterogeneity of the My samples, even after several filtrations and/or centrifugations, it was impossible to obtain satisfactory HPLC conditions for direct monitoring. Therefore, the assays were repeated with crude CM fractions in which direct observation of the cymoxanil was possible by reference to the calibration curves. In both the L and N strains, the cymoxanil concentration is *ca.* 50% ($2–3 \cdot 10^{-4}$ *M*, Fig. 6) of the initial concentration ($6 \cdot 10^{-4}$ *M*, Fig. 4). As expected, this implies partition of cymoxanil between the CM and the My compartments. Cymoxanil is stable in aqueous solution as well as under UV light conditions (254 nm) within several hours [15]. It is relatively stable in the tolerant N strain, losing only 13% of its initial concentration after 6 h. However, it is rapidly metabolized in the sensitive L strain, losing up to *ca.* 80% of its initial concentation after 6 h (Fig. 6).

These observations agree with other studies [1,3] and with our previous results concerning the *in situ* ^1H NMR monitoring of cymoxanil in the two strains [6,15].

This first approach was carried out in order to test the possibilities of the direct IRSP-HPLC monitoring in plant biological media, and so we did not focus on the limits of detection of cymoxanil. Nevertheless, Fig. 6 shows that a concentration

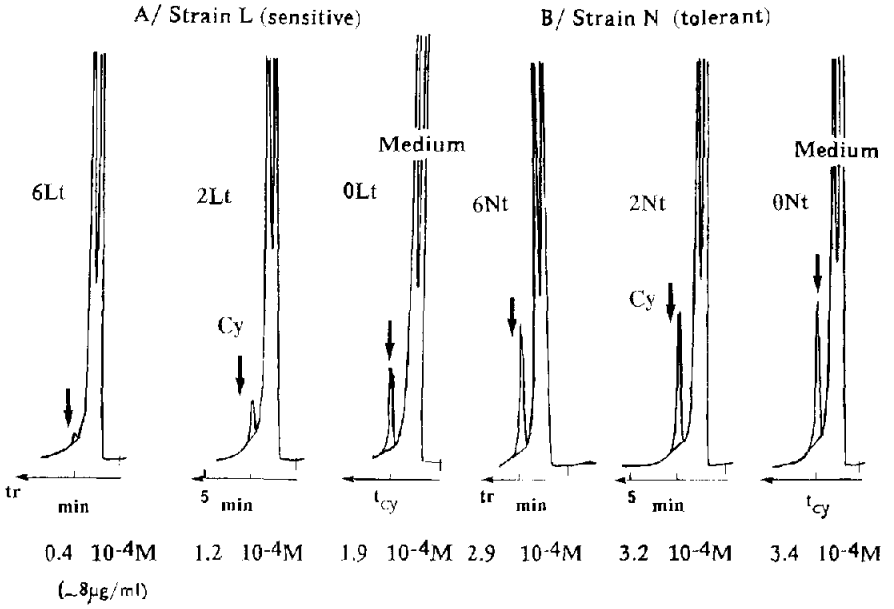


Fig. 6. *In situ* monitoring of cymoxanil in *B. cinerea* culture medium (CM) using ISRP-HPLC. Chromatographic conditions: column packing, Pinkerton GFF ISRP (Regis) 5 µm; guard column, 10 mm × 3 mm I.D.; analytical column, 50 mm × 4.6 mm I.D. Isocratic elution with phosphate buffer (0.1 *M*, pH 7.0) and 3% THF–isopropanol (6:10, v/v) at a flow-rate of 0.6 ml/min. After centrifugation, 5-µl aliquots of treated *B. cinerea* CM were injected. The UV detector was set at 254 nm (0.5 a.u.f.s).

as low as 8 µg/ml may be detected in the 6Lt sample, but owing to the low sensitivity of measurements in these experiments, the limit of cymoxanil detection is probably better than 8 µg/ml.

Behaviour of cymoxanil in arthropod haemolymph

The behaviour of cymoxanil was also analysed by using the same ISRP-HPLC approach in animal media, such as lobster (*H. americanus*) or locust (*L. migratoria*) haemolymphs: these media are known to cause the enzymic hydrolysis of xenobiotic compounds [5,6,8].

The efficient metabolism of cymoxanil in these media was demonstrated by the decrease in its concentration over 6 h. In order to reveal the possible presence of the metabolite(s) M_1, M_2 etc., which are more polar than cymoxanil, the organic modifiers were eliminated from eluent, and the detection conditions were modifed (absorption at 230 nm). Under such isocratic conditions, the cymoxanil had a k' value of 2.28, and in the case of the lobster haemolymph (Fig. 7A), two new peaks appeared progressively; their capacity factors (k') of 1.57 and 0.85 are intermediate between those of the biological medium and of cymoxanil. The chromatograms obtained using locust haemolymph also indicated rapid metabolism at 35°C, but were rather complex owing to the presence of peaks due to lipid

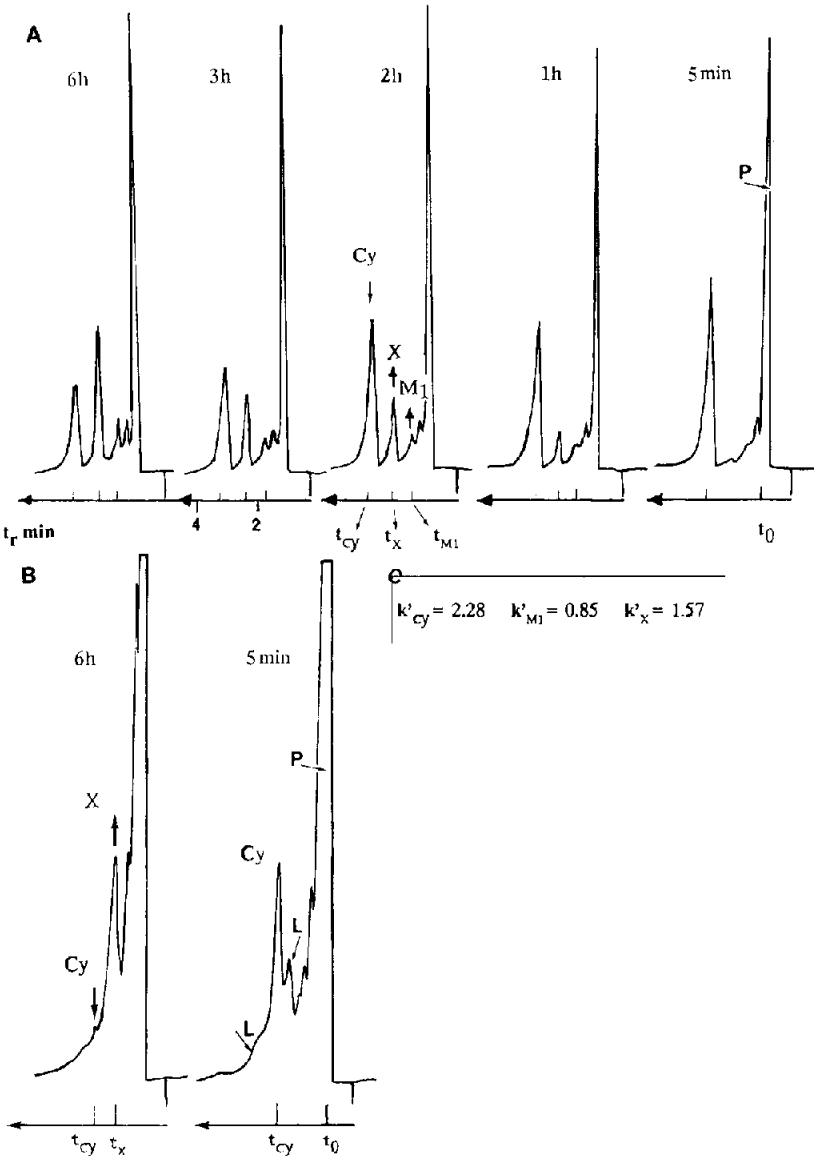


Fig. 7. *In situ* monitoring of cymoxanil in arthropod haemolymph using ISRP-HPLC: (A) in *H. americanus* haemolymph (at 23°C); (B) in *L. Migratoria* haemolymph (at 35°C). Chromatographic conditions: (A) columns as in Fig. 6; isocratic elution with phosphate buffer (0.1 *M*, pH 7.2) at a flow-rate of 0.4 ml/min; after centrifugation, 5-μl aliquots of an incubation mixture (1:1, v/v) of lobster haemolymph with a $1.4 \cdot 10^{-3}$ *M* cymoxanil water–THF (90:10, v/v) solution were injected; the UV detector was set at 230 nm (0.5 a.u.f.s.); peak P = lobster haemolymph proteins; (B) columns and elution conditions as in (A); after centrifugation 5-μl aliquots of an incubation mixture (1:1, v/v) of locust haemolymph with a $1.4 \cdot 10^{-3}$ *M* cymoxanil water–THF (90:10, v/v) solution were injected; peak P = locust haemolymph proteins; peak L = lipid components of locust haemolymph.

components. This situation entailed more difficult ISRP monitoring (Fig. 7B), so we turned our attention to lobster haemolymph.

The metabolite M_1 was synthesized as described previously (Fig. 3) [1]. The corresponding ^1H NMR, ^{13}C NMR and CI-MS/NH_4^+ spectra were consistent with the assigned structure (Fig. 2) [15]. By HPLC overloading, M_1 was identified as the compound with a k' value of 0.85, *i.e.* the apparently less abundant of the two metabolites formed (Fig. 7A).

The predominant metabolite X ($k' = 1.57$, Fig. 7A) was isolated after preliminary extraction and TLC (Fig. 5). This compound was confirmed as metabolite *X* ($k' - 1.57$) by HPLC overloading, and the corresponding ^1H NMR, ^{13}C NMR and NH_4^+ CI-MS spectra indicated an isomeric structure of cymoxanil. Moreover, the absence of the ν(CN) vibration in IR spectra suggested a cyclic structure, such as a 2,4-pyrimidinedione (Fig. 2) [2,15]. The differences observed in CI/NH_4^+ mass spectra for the two isomers agree with such a hypothesis, since chelation in cymoxanil would be strong enough to explain the formation of a significant adduct ion, but it is very weak in the cyclic X isomer (Fig. 2).

Metabolism of cymoxanil leading to both X and M_1 metabolites was also demonstrated by a direct ^1H NMR study of locust and lobster haemolymph [15].

CONCLUSIONS

Direct ISRP-HPLC monitoring provides a good approach for studying the *in situ* behaviour of xenobiotic cymoxanil in the culture medium (CM) fraction of *B. cinerea*. The validity of the method is confirmed by the observation of the same differences between the strains L and N as in a more expensive and sophisticated method employing radiolabelled cymoxanil.

Moreover, the method described here reveals the isomerization of cymoxanil in lobster haemolymph into isomer X. Further experiments are in progress and should help us to ascertain the structure of X and to specify its role in the metabolic pathway of cymoxanil, in locust or lobster haemolymph, as well as in *B. cinerea*. These metabolites also need to be quantified in order to establish the relative extent of each metabolic process.

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