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Radio-high-performance liquid chromatography for ecotoxicity assessment of insect growth regulators

Richard Tykva [a,*], Věra Vlasáková [b], Jaroslav Novák [a], Libor Havlíček [b]

[a] *Institute of Organic Chemistry and Biochemistry, Academy of Sciences, CZ-16610 Prague 6, Czech Republic*
[b] *Institute of Experimental Botany, Academy of Sciences, CZ-14220 Prague 4, Czech Republic*

Abstract

The described radiochromatographic method permits fast and high-sensitivity monitoring of soil biodegradation products of an insect growth regulator for its environmental risk assessment. We analyzed and compared two diastereoisomers of ethyl *N*-(2-{4-[(2-hydroxycyclohexyl) methyl]phenoxy}ethyl)carbamate, namely its *cis*-(1*S*,2*S*) isomer JN-W330 and a *trans*-(1*R*,2*S*) isomer JN-W331. Microbial conversion of the *cis*-isomer to the *trans*-isomer was proved by mass spectrometry analyzer. Among the chromatographic columns tested, the best separation was found with a 125 mm × 4 mm i.d. column packed with Supersphere 100 RP-C_{18}, 5 μm and an acetonitrile–water gradient. The detection limit for the both isomers was in the range of 120–250 Bq (0.3–0.8 ng) at a concentration of 2 ng/ml with radiometric detection. The calibration curves for standard solutions were linear in the range of 150 Bq–150 kBq ($r = 0.996$). The method enabled us to compare the analyzed juvenoids with biologically active oostatic peptides in terms of their environmental safety.

Keywords: Insect growth regulators; Radiochromatography; Biodegradation

1. Introduction

The current prices of agricultural plant products can be markedly affected by efficient methods of plant protection. An important part of this protection is the control of harmful insects. At present, the most frequent means of plant protection against insects is the insect growth regulators (IGRs), which are commonly summarily termed pesticides [1]. The selection of a suitable IGR should take into account not only its biological activity, but also its environmental safety.

During application of IGR on plants, part of the agent usually falls on the soil surface. Its subsequent penetration into the subsurface environment can cause pollution of soil, sediment and ground water. Evaluation of the corresponding ecotoxicity of IGR should take into consideration, in addition to the actual agent used, also its degradation products arising for the most part as metabolites of soil aerobic microorganisms [2].

Our study describes a procedure based on high-performance liquid radiochromatography, which permits a high-sensitivity detection of radiolabelled products of soil IGR degradation and a subsequent use of individual isolated fractions for toxicity assay. The high sensitivity of the radiometric detector was given by the independence of its response on chemical structures of the analyzed compounds. The method was used for monitoring the degradation of radiolabelled diastereoisomers of a bioanalog of a juvenile hormone (juvenoid) by a soil microbial isolate. Juvenoids and their analogs have been known since the seventies and are still used in wide practice because of their high efficiency [3]. The two diastereoisomers were chosen for their relatively high biological activity relative to corresponding racemic substances and another pair of diastereoisomers. The biological activity was assessed in screening tests on the pupae of the yellow mealworm (*Tenebrio molitor*) [4]. The use of biologically active isomers ensures a reduced environmental load as compared with the use of a racemic mixture. The course of the microbial degradation of the two isomers determined by radio-HPLC, along with their previously determined biological activities, allows us to determine which of them is more useful in terms of application. A method

* Corresponding author. Tel.: +420-220-183-565; fax: +420-233-331-714.
E-mail address: tykva@uochb.cas.cz (R. Tykva).

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developed by us recently [5] was chosen for simple toxicity comparison.

The procedure described in this paper offers a potentially widely applicable methodological approach to the topical problem of chiral environmental pollutants [6].

Using the described methodical approach, the comparison of environmental safety of the analyzed juvenoids and a biologically active peptide was carried out to demonstrate the advantages of peptides as a new potential type of IGRs.

The methodical approach described in this paper is based on HPLC separation of biodegradation products of a pesticide using two types of detectors. A radiometric detector was applied for measurement of radioactivity of tracer amounts of biodegradation products within a complex sample while an UV detector made subsequently possible to isolate these non-radioactive products for comparison of their toxicity.

2. Experimental

2.1. Reagents

Two diastereoisomers (Fig. 1) of ethyl *N*-(2-{4-[(2-hydroxycyclohexyl)methyl]phenoxy}ethyl)carbamate, namely a *cis*-(1*S*,2*S*) isomer JN-W330 and a *trans*-(1*R*,2*S*) isomer JN-W331, were prepared by an enzymatic reduction mediated by *Saccharomyces cerevisiae* [7]. The compounds were obtained with respective enantiomeric purity values e.e. = 79% (JN-W330) and e.e. > 99% (JN-W331). A common exchange reaction yielded radiochemical purity higher than 98% and molar ^{3}H activity of 88.3 GBq/mmol for JN-W330 and 61.8 GBq/mmol for JN-W331. The radiolabelled compounds were stored in ethanol (UV-spectroscopic purity) in glass ampoules at −18 °C. The absence of radiolysis was checked before each experiment.

Methanol and acetonitrile (HPLC gradient grade) and ethanol (UV-spectroscopic purity) were purchased from Merck. All other reagents were of analytical grade (Lachema, Brno, Czech Republic).

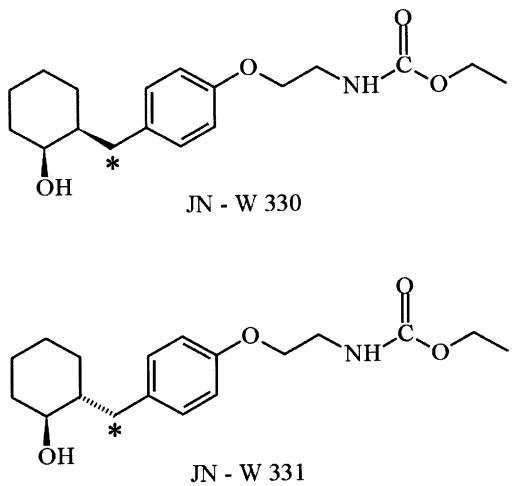

Fig. 1. Chemical structures of the analyzed juvenoid diastereoisomers. The star indicates ^{3}H position.

2.2. Radio-HPLC

Radio-HPLC measurements were performed on a liquid chromatograph (Waters) with two detection systems connected on-line to a programmable multi-wavelength UV detector (Waters, model 490E) and a radiometric flow-through detector (Beckman, model 171). The latter detector was used together with a solvent delivery module for scintillator pumping to measure radioactivity by the admixture method. After passing through the UV detector the column effluent was continuously mixed in an attached mixer with the liquid scintillator Ready Safe (Beckman) in a ratio of 1:2.5. The mixture was passed through a detection cell having a volume of 1 ml. The threshold of detection was set at 0.02% and the residence time was 0.3–0.42 min. The absolute detection limit in this system, defined by a signal-to-noise ratio of 3, was in the range of 120–50 Bq for both isomers (at a concentration of 2 ng/ml).

The four following stainless steel analytical columns were tested at laboratory temperature for the separation of radiolabelled soil biodegradation products: Lichrosphere 100 (5 μm, 125 mm × 4 mm, Hewlett-Packard), Supersphere 100 RP-18 (4 μm, 125 mm × 4 mm, Merck), Hypersil BDS C_{18} (3 μm, 75 mm × 4 mm, Hewlett-Packard), Elipse XDB C_{18} (3.5 μm, 75 mm × 4 mm, Hewlett-Packard). All columns were protected by a 4 mm × 4 mm guard column with the same packing as the analytical column. A Rheodyne injector was used for sampling. A number of gradient and isocratic procedures were tested using double-distilled water and acetonitrile. The best separation was achieved with the Supersphere 100 RP-18 column, with a linear gradient programme (water–cetonitrile, 80:20 (v/v), increasing to 65:35 (v/v), over 15 min) followed by an isocratic regime for 15 min. The flow rate used was 0.8 ml/min, the UV detector wavelength was set at 230 nm, 0.05 AUFS.

2.3. Sample preparation for radio-HPLC

The soil isolate, a strain of *Candida* sp. yeast, was used for the model study of biodegradation [8] of the two diastereoisomers. The strain was microscopically checked prior to each experiment. A volume of 60 ml of a culture grown overnight at 28 °C in a minimal medium (Difco Yeast Nitrogen Base w/o amino acids) with 3% (w/w) Difco vitamin-free Casamino acids and 1% (w/w) glucose was centrifuged for 5 min at 10^4 *g*, washed with 450 mM phosphate buffer (pH 7.1) and resuspended in the same buffer (30 ml). For radio-HPLC, the ^{3}H-labelled diastereoisomers (JN-W330 1.34 μg; 370 kBq or JN-W331 1.92 μg; 370 kBq, respectively) were added to the cell suspension (2 ml). The cells with the added isomer as the only carbon source were shaken at 28 °C for different time intervals.

During the cultivation, pH was controlled and adjusted to pH 7.1 by addition of NaH_2PO_4 when needed. The volume was adjusted by sterile water. The mixture was centrifuged and the supernatant was extracted with diethyl ether (five times 25 ml). Pooled extracts were evaporated under reduced pressure. The residue was dissolved in methanol (5 ml). A 200 μl volume was subjected to radio-HPLC. The effectiveness of the extraction procedure measured by radioactivity was 86.8%.

2.4. MS analysis

A micromass quadruple mass spectrometry analyzer (Waters, model ZMD 2000) was used for MS analyses. The samples were injected by direct infusion using a Harvard syringe pump (50 μl/min). The compounds were analyzed either by the position electrospray ionization (ESI^+) technique or atmospheric pressure chemical ionization $APCT^+$ technique. Nitrogen was used as nabulizing gas. ESI^+: capillary voltage 3.2 kV, source block temperature (SBT) 80 °C, desolvation temperature (DST) 120 °C, sampling cone voltage (CV) 15–30 V. $APCT^+$: corona discharge pin 3.2 kV, SBT 80 °C, DST 420 °C, CV 15–30 V. Preliminary data acquisition was performed using JN-W330 as a reference sample in full scan in a positive mode from 90 to 600 *m/z*.

2.5. Ecotoxicity comparison of the isomers and their biodegradation products

The main goal of this work was to develop radio-HPLC analysis making possible to prepare components for assessment of total pesticide toxicity in the subsurface environment. Besides the applied pesticide, their soil biodegradation products [2] has to be considered. To demonstrate application of the obtained chromatographic fractions in comparison of ecotoxicity of JN-W330 and JN-W331, the recently described [5] luminescence assay of the ATP/ADP content of human cells to various concentrations of the isolated fractions was used.

2.6. Isolation of non-radioactive biodegradation products

The non-radioactive biodegradation products are necessary for MS analysis and ecotoxicity comparison. For these measurements, the sample preparation for radio-HPLC was modified as follows. A culture volume was increased to 150 ml and sterile water was used instead of phosphate buffer. To 5 ml of the cell suspension containing a radiolabelled isomer (3.35 μg of JN-W330 or 4.79 μg of JN-W331, respectively), 3 mg of the same non-active isomer were added. The mixtures were shaken 3 days. The individual fractions were collected according to the retention values obtained from the radioanalysis and corrected by the value of the time delay between the UV and the radiometric detector. This delay in the used system was 1.2 min. The fractions were collected five or six times into the glass tubes, evaporated to dry and stored at −18 °C.

3. Results and discussion

3.1. Radio-HPLC

Typical radiochromatogram of extracts of JN-W330 and its biodegradation products (**a**–**e**), generated during 3-days incubation with the model microbial soil isolate, is shown in Fig. 2. In the corresponding radiochromatogram of JN-W331 only peaks (**a**–**c**) were found.

The radiochromatograms for the both isomers were obtained after 1 and 3 days of incubation. Each peak was evaluated as a ratio of its counting rate (cpm) to the totally measured cpm in all peaks of the appropriate radiochromatogram (relative concentration c_{rel} in percentage). Precision of these evaluations (Table 1) was expressed by means of CV from four experiments. This value ranged from 1.7 to 5.1%.

The values of retention time of the measured peaks are given in Table 2. The total run time was 35 min.

The obtained results showed a significantly higher biodegradation level of JN-W330. Comparison of the proportion of degradation products of the two isomers (Table 1) showed that more than 20% of the *cis*-isomer was degraded after a 1-day treatment, forming five radioactive fractions (Fig. 2). On the other hand, only about 2% of the *trans*-isomer was degraded during the same time period (Table 1), forming three qualitatively equal fractions in the polar and semi-polar area (**a**–**c**). During the next two incubation days the degradation levels of the isomers changed only marginally: 22.0–21.2% for *cis*-isomer and 2.2–3.6% for *trans*-isomer (Table 1).

Radio-HLPC of samples prepared analogously to the samples with radioactive juvenoids, but without the *Candida* sp. microbial strain, was used to measure the influence of

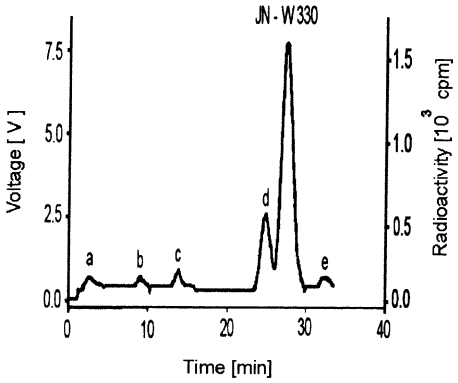

Fig. 2. Radiochromatogram of the biodegradation products of ^{3}H-labelled *cis*-isomer JN-W330 after 3-days incubation. Chromatographic peaks (**a**–**e**), indicate biodegradation products. HPLC conditions (see Section 2.2).

Table 1
Comparison of biodegradation of juvenoid diastereoisomers by *Candida* sp.

Isomer	Incubation period (days)	Relative concentration[a] c_{rel} (%)						Total degradation of isomer	
		a	**b**	**c**	**d**[b]	**e**	JN-W330	c_{rel} (%)	R.S.D. (%, $n = 4$)
JN-W330	1	1.3	2.9	3.5	2.7	11.6	78.0	22.0	4.1
	3	1.8	3.1	4.4	5.7	6.7	78.8	21.2	1.9
JN-W331	1	0.7	0.9	2.3	96.1	n.d.[c]	–	2.2	3.9
	3	0.6	1.1	1.9	96.4	n.d.[c]	–	3.6	5.3

[a] c_{rel} are given as mean values.
[b] **d** was identified as JN-W331.
[c] n.d., not detectable.

abiotic degradation on the results. No degradation products were found in either of the two isomers. Besides, the mixture of the growth medium residues, yeast cells and other undesirable compounds was proved to be non-toxic even in high concentrations (of the order of mg/ml).

The absolute detection limit in the system, defined by a signal-to-noise ratio of 3, was in the range of 120–250 Bq (0.3–0.8 ng) at a concentration of 2 ng/ml for both isomers. The peaks on the radiochromatograms were symmetrical without tailing. The calibration curves of standard solutions of known amounts of the isomers standards were linear in the range of 150 Bq–150 kBq ($r = 0.996$).

The recovery following the extraction procedure was determined by comparing the starting radioactivity injected into the system with that of the extracted sample from incubation procedure. The mean analytical recoveries of both isomers ranged from 70.3 to 76.8%.

3.2. Microbial conversion of isomers

The MS analysis was first used to determine the pseudomolecular ions of the two pure isomers. Afterwards, the procedure was used for the **d** fraction (Fig. 2) arising solely during the degradation of the *cis*-isomer (Table 1) in the position of the *trans*-isomer standard. After isolation, the compound was filtered, freeze-dried and finally dissolved in methanol acidified by formic acid for use in mass detector. Using the ESI^+ ionisation technique, we determined the $[M+H]^+$ *m/z* 322.2 and the $[M+Na]^+$ *m/z* 344.2 ions. The $[M–H_2O+H]^+$ *m/z* 304.2 and $[M+H]^+$ *m/z* 322.2 ions were determined by means of $APCT^+$ ionization (Fig. 3). The **d** fraction showed spectral values equal to the *trans*-isomer providing thus an evidence of the microbial conversion of the *cis*-isomer to the *trans*-isomer.

Table 2
Values of retention time t_R of radiochromatographic peaks

	a	**b**	**c**	**d**[a]	**e**	JN-W330
Mean t_R	1.62	11.89	16.36	27.09	33.46	28.74
±S.D.	0.04	0.33	0.25	0.42	0.55	0.32

[a] **d** was identified as JN-W331.

3.3. Ecotoxicity comparison

In terms of ecotoxicity, the *trans*-isomer shows a significantly lower toxicity than the *cis*-isomer (Fig. 4). The toxicity of the mixture of both degradation products in the semi-polar area (**b** and **c**, Fig. 2) is lower than the toxicity of the *trans*-isomer (Fig. 4). The most polar product (**a**, Fig. 2), which we managed to acquire only in a limited amount (Fig. 4), showed the significantly lowest toxicity. In view of the course of the biodegradation of the two isomers (Table 1), the total toxicity of the *trans*-isomer is lower compared to the *cis*-isomer, which showed a higher biological activity [4]. Under the experimental conditions used, each of the two isomers thus meets only one of the basic requirements of higher biological efficiency and lower environmental hazard.

Recently, it was proved that a separate application of a bacterial soil isolate or a mold mixture, respectively, influence biodegradation course by *Candida* sp. [9]. Therefore, due to the found influence of biodegradation fractions on the total ecotoxicity of the analyzed diastereoisomers (Fig. 4), application of the described radio-HPLC for a sample at the output of a soil column [10] is recommended for the next

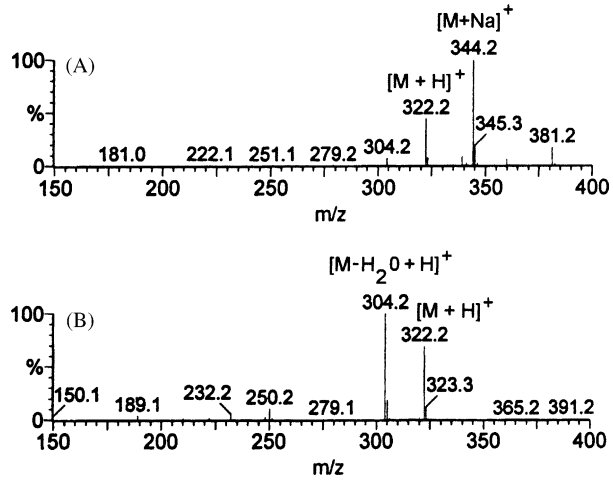

Fig. 3. MS spectra. (A) ESI^+ spectrum of the isolated fraction **d** (Fig. 2); (B) $APCI^+$ spectrum of the same fraction.

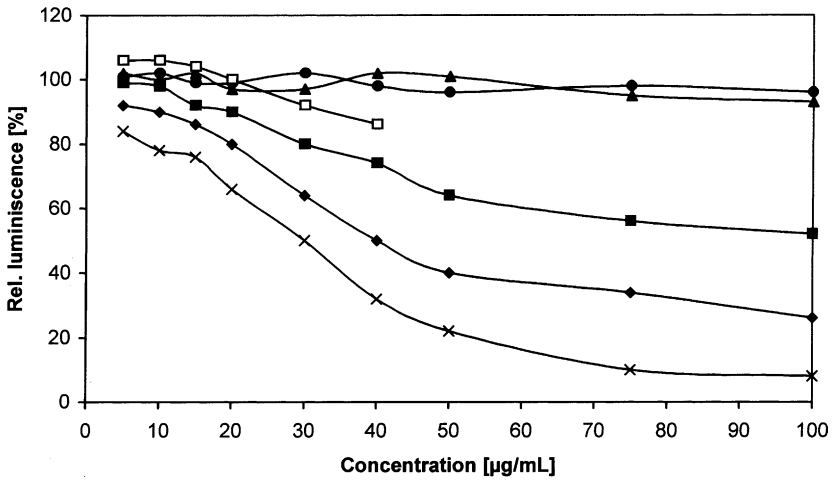

Fig. 4. Ecotoxicity expressed as a relative luminescence for different concentrations of the analyzed compounds. (×) *cis*-Isomer, (◆) *trans*-isomer, (■) ***b*** + ***c*** fractions (Fig. 2), (□) **a** fraction (Fig. 2), (●) tetrapeptide H-Tyr-Asp-Pro-Ala-OH, (▲) degradation product of the tetrapeptide (Asp-Pro).

study. Simultaneously, further analytical tools should be applied for evaluating toxicity of the chromatographic fractions.

3.4. Comparison of ecotoxicity of oostatic peptides

For the sake of comparison, Fig. 4 gives values for selected biologically active analog H-Tyr-Asp-Pro-Ala-OH of a natural insect decapeptide [11] and its only long-term degradation product Asp-Pro [12], both dissolved in methanol. The peptides exhibited negligible toxicities. Since the tetrapeptide has a proven oostatic activity [11] and is easy to prepare, such compounds represent a prospective alternative of the IGRs.

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