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Separation and aquatic toxicity of enantiomers of the organophosphorus insecticide *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN)

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ABSTRACT

Enantioselectivity in separation and toxicity of chiral pesticides has become important research areas in environmental science, because these studies give a deeper insight into the environmental effect of chiral pesticides. In this study, enantiomeric separation of the organophosphorus pesticide and acaricide *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN) was investigated by chiral high-performance liquid chromatography (HPLC) with two chiral stationary phases. The racemate and separated enantiomers of EPN were tested for aquatic toxicities assay using *Daphnia magna* and zebrafish (*Danio rerio*) embryo test. The enantiomers of EPN were completely separated on Chiralpak AD and Chiralpak AS columns coupled with a circular dichroism detector at 236 nm. Better separations were achieved with lower temperatures (e.g., 20 °C) and lower levels of polar modifiers (e.g., 1%). A significant difference was found between the enantiomers in their acute aquatic toxicity; the (+)-enantiomer was about 10 times more toxic than its antipode. On the contrary, the (−)-enantiomer induced crooked body, yolk sac edema and pericardial edema significantly more than (+)-enantiomer in the zebrafish embryo test. These results suggest that biological toxicity of chiral pesticides should be assessed by using their individual enantiomers with more comprehensive methods.

1. Introduction

Chirality can be commonly found in organophosphorus pesticides (OPs), which were widely used to protect crops and livestock from being infested by pest in the past 60 years (Prelog, 1976). At present, all chiral OPs are still used in their racemic forms because of high costs in industrial scale preparation of enantiomers. However, many studies have indicated that enantiomers of chiral OPs usually differ in biological effects such as acute toxicity, endocrine disruption effects and fate in the environment (Kohler et al., 1997; Lewis et al., 1999; Buser et al., 2002; Liu et al., 2005a,b; Garrison, 2006). For example, the (−)-enantiomers of fonofos and profenofos were about 10 times more toxic to *Ceriodaphnia dubia* and *D. magna* than their corresponding (+)-enantiomers (Liu et al., 2005b). For some chiral OPs, only one enantiomer has biological activity toward target organisms, the other enantiomer shows less effect, but may have adverse effects on some nontarget organisms in the environment. The key of assessing enantioselectivity of chiral OPs is the preparation and analysis of enantiomers. Although the organophosphorus pesticides and acaricide *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN) has been widely used for agricultural purpose in many countries because of its low cost and broad spectrum activity, the aquatic toxicity of EPN enantiomers have not been studied. However, enantiomer standards of chiral OPs are not available at the present time. Fortunately, as chiral stationary phases (CSPs) have rapidly developed in the past decades, high-performance liquid chromatography (HPLC) coupled with circular dichroism (CD) has become a powerful tool for the separation, analysis and small scale preparation of individual enantiomers of chiral pesticides. Enantiomers of some chiral OPs with a single chiral center at phosphorus, carbon, or sulfur have been successfully separated by HPLC on polysaccharide or Pirkle mode CSPs (Gao et al., 1997; Ellington et al., 2001; Wang et al., 2006).

Furthermore, the toxicity assay using zebrafish embryo has been increasingly used in toxicological studies (Ensenbach, 1998; Beis and Stainier, 2006). A number of biochemical and molecular mechanisms could specifically influent at the level of cells, receptors, tissues, and organs during embryogenesis. In recent studies, zebrafish embryo has been proved to be a useful model in evaluating enantioselective toxicity of chiral pesticides (Xu et al., 2008a,b). It can offer more useful toxicological information of chiral pesticides.

Therefore, it is imperative to assess the environmental safety of chiral OPs using their enantiopure forms instead of racemate. In this study, enantioseparation of EPN was investigated using HPLC on four chiral columns (Chiralpak AD, Chiralpak AS, Chiralcel OD, Chiralcel OJ) with different composition of mobile phase and

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temperature. Enantiomers of EPN were characterized by CD and GC–MS. Pure enantiomers of EPN were collected at the outlet of HPLC under the baseline separation condition and then used to evaluate the enantioselective toxicity with *D. magna* and zebrafish embryo.

2. Materials and methods

2.1. Reagents and chemicals

Insecticide EPN (racemate, purity of 99%) was purchased from Sigma (USA). All solvents (*n*-hexane, ethanol [EtOH], isopropanol [IPA], acetone) used in this study were of HPLC grade and purchased from Tedia (Fairfield, OH, USA). Other chemicals were all analytical standards from local chemical factories. The insecticide stock solution of 2000 mg L^{-1} was prepared by dissolving EPN in *n*-hexane and stored at 4 °C in darkness. Working solutions of the pesticide were prepared daily by diluting the stock solution in *n*-hexane.

2.2. Chromatographic separation and analysis

Enantiomeric separation was performed on a Jasco LC-2000 series HPLC system (Jasco, Tokyo, Japan) composed of a PU-2089 quaternary gradient pump, a mobile phase vacuum degasser, an AS-1559 autosampler with a 100-μL sample loop, a CO-2060 column temperature control compartment, a UV-2075 plus UV/vis detector, a variable-wavelength CD-2095 CD detector, and a LC-NetII/ADC data collector. Chromatographic data were recorded and processed with the ChromPass software (Jasco, Tokyo, Japan). The chiral columns used in this study were Chiralpak AD [amylase *tris*-(3,5-dimethylphenyl-carbamate)], Chiralpak AS [amylase *tris*-((S)-1-methyl-phenyl-carbamate)], Chiralcel OD [cellulose *tris*-(3,5-dimethylphenyl-carbamate)] and Chiralcel OJ [cellulose *tris*-(4-methylbenzoate)]. All the columns (250 × 4.6 mm i.d., 5-μm) tested were purchased from Daicel Chemical Industries (Tokyo, Japan). For chiral separation in normal-phase mode, a volume of 20 μL (200 mg L^{-1}) of sample was injected. *n*-Hexane was the mobile phase, and EtOH or IPA was added as the polar modifiers. The wavelengths of the UV and CD detectors were set at 236 nm. Chromatography was performed at room temperature (25 ± 2 °C). The effect of temperature to the separation was determined over the range of 20–40 °C.

Considering the result of initial method development, Chiralcel AS column was chose to prepare the individual enantiomers which were used for the subsequent toxicity assays. The separated enantiomers were manually collected into separate glass vials at the HPLC outlet. The solvents were then evaporated under a nitrogen stream to dryness and the enantiomers were redissolved in acetone. For the analysis of purity of enantiomers by HPLC, conditions were the same as for preparative chiral separation. The purity was found to be ⩾99.9% for each separated enantiomer in this study, and no racemization was observed during the experiment. An Agilent 7890A GC system interfaced with an Agilent 5975C mass detector equipped with a HP-5MS fused silica capillary column (30 m × 0.25 mm ID, film thickness 0.25 μm) was used for concentration analysis. Helium was used as the carrier gas with a flow rate of 1.0 mL min^{-1}. The inlet temperature was 230 °C, the interface was 280 °C, and the oven temperature was held at 210 °C.

2.3. Identification of enantiomers

The HPLC-CD technique was used for distinguishing between the enantiomers of EPN according to the CD signal of (−)- or (+)-enantiomer. At the top of the chromatographic peak, the CD spectra were recorded over the wavelength range of 220–420 nm by using the stop-flow method. The CD spectra were detected with a resolution of 0.2 nm, a 10× accumulation, a set of fast responses, and a scan speed of 4 nm s^{-1}. At the beginning of CD scanning of the separated enantiomers, the background CD spectrum of the mobile phase was recorded and automatically subtracted from the spectrum of each peak.

2.4. Acute aquatic toxicity

The aquatic toxicity of individual EPN enantiomers and racemate were evaluated through 24-h and 48-h acute toxicity assays using *D. magna*. The test animals were originally obtained from the Chinese Academy of Protection and Medical Science (Beijing, China). The procedures of the test followed the EPA guidelines (US EPA, 2002). Briefly, 20 mL of blank or test solutions were transferred to 50 mL glass beakers. Four replicates were prepared for each concentration level and blank (0, 0.5, 2, 8, 16, 32 μg L^{-1}). Five active *D. magna* aged 6–24 h were added into each beaker. The sensitivity of *D. magna* to potassium dichromate ($K_2Cr_2O_7$) was performed as a positive control by the International Organization for Standardization (ISO) method (UNEP/WHO, 1996). Culture medium M4 maintained at 20 ± 1 °C was used to constitute the solutions (OECD, 1997). The test organisms were fed with *Scenedesmus obliquus* 6 h before the exposure. All vials were monitored at 24-h intervals for the 48-h exposure period. The concentration that caused 50% mortality (LC_{50}) of the test population was determined by probit analysis using Origin 8.0 (MA, USA).

2.5. Embryo exposure and toxicity assays

Zebrafish embryos were collected and prepared under appropriate fish-mating conditions as that of Westerfield (1993), and used for the exposure experiments immediately. The embryo toxicity test was conducted by OECD test guidelines (Braunbeck and Lammer, 2006). Briefly, the eggs were placed to exposure chambers which contain culture medium spiked with different known concentrations of chemicals and cultured for 60 min. Fertilized eggs were separated from the nonfertilized ones and transferred to 24-well plated (Krackeler Scientific, Albany, NY, USA) by a pipet. The culture medium contained 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 4.2 mM $NaHCO_3$ in ultrapure water (18.3 MΩ cm resistivity) produced by Milli-Q system (Millipore, MA, USA). To eliminate interference, 20 wells in each plate contained only one embryo in 2 mL of culture medium with the same concentration of EPN, while the remaining four wells of each plate served as internal controls which contained only artificial water with 0.5% acetone. Five concentrations were tested (0.05, 0.2, 0.4, 0.8, 1.0 mg L^{-1}), while three plates were used as replicates for each concentration. The plates were covered to prevent possible water evaporation and carried out at 20 ± 1 °C with constant lighting. The medium was changed daily.

The development of embryos from blastula to early larval stage was monitored at specific time points (12, 24, 36, 48, 60, and 72 h). Endpoints used for assessing the effects of EPN enantiomers included embryo mortality, yolk sac edema, pericardial edema and crooked body.

3. Results and discussion

3.1. Separation and analysis of enantiomers

Enantiomeric separation of EPN was initially evaluated on four kinds of columns with different CSPs. Baseline resolution for the two enantiomers was obtained on the Chiralpak AD column and