Journal of Chromatography A, 1452 (2016) 116–120

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Separation, isolation and stereochemical assignment of imazalil enantiomers and their quantitation in an *in vitro* toxicity test

Mònica Escolà Casas[a], Andreas Christopher Kretschmann[b], Lars Andernach[c], Till Opatz[c], Kai Bester[a,*]

[a] Environmental Science, Aarhus University, Frederiksborgvej 399, 4000 Roskilde, Denmark
[b] Section of Analytical Biosciences, Department of Pharmacy, University of Copenhagen, Universitetsparken 2, 2100 København Ø, Denmark
[c] Institute of Organic Chemistry, Johannes Gutenberg-University, Duesbergweg 10-14, 55128 Mainz, Germany

ARTICLE INFO

Article history:
Received 18 January 2016
Received in revised form 26 April 2016
Accepted 3 May 2016
Available online 4 May 2016

Keywords:
Imazalil
Enantiomers
HPLC-UV
Isolation
Vibrational circular dichroism
HPLC–MS/MS

ABSTRACT

A simple method for the separation of the enantiomers of the fungicide imazalil was developed. Racemic imazalil was separated into its enantiomers with an enantiomeric purity of 99% using HPLC-UV with an enantioselective column (permethylated cyclodextrin) operated in reversed phase mode (water with 0.2% trimethylamine and 0.08% acetic acid and methanol). The absolute configuration of the separated enantiomers was assigned and unequivocally confirmed by optical rotation as well as by vibrational circular dichroism (VCD) and electronic circular dichroism (ECD) combined with ab-initio calculations. The same enantioselective column was also used to develop an HPLC–MS/MS method for the quantification of imazalil enantiomers. The HPLC–MS/MS method reached limits of quantification (LOQs) of 0.025 mg/mL with 5 μL injections. This method was used to verify imazalil concentrations and enantiomeric fractions in samples from an *in vitro* test on effects on human steroidogenesis (H295R steroidogenesis assay). The quantification verified the stability of the enantiomers of imazalil during the *in vitro* tests.

1. Introduction

Azole fungicides have been detected in surface waters in concentrations up to 9.1 μg/L [1]. Recent studies indicate that exposure to azole fungicides might be of concern for both humans and wildlife due to their potential to disrupt the endocrine system in mammals [2,3] or to synergize the toxicity of other pesticides in aquatic organisms [4]. Although 30% of the organic chemical pesticides on the market are chiral [5,6] and although it is known that enantiomers exhibit substantial differences in their pesticidal activities [7], hardly any information is available on which enantiomeric form is mainly responsible for adverse side effects.

The enantiomers of a compound may interact differently with other chiral molecules (*e.g.* enzymes), resulting in different degradation rates and thus a different persistence in the environment [8]. Differences in metabolism are probably the most significant stereo-selective factor [9]. At the same time, toxicity can be stereoselective due to differences in toxicokinetics (*e.g.*, metabolism) and toxicodynamics [9].

Imazalil or 1-[2-(allyloxy)-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole is a commonly used azole fungicide in agriculture. Imazalil is *e.g.* used for post-harvest treatment of citrus fruits and bananas, [10,11]. Imazalil contains a stereogenic center (Fig. 1) and its efficiency depends on the absolute configuration [12]. While its degradation in soil is not enantioselective [13], it can be enriched enantioselectively on orange surfaces [14].

Enantiopure imazalil is not commercially available. Thus, its enantiomers had to be isolated from racemic imazalil before enantioselective analysis and toxicological determinations [8]. Additionally a sensitive and selective method for verification of enantiomeric composition in toxicity experiments (*i.e.* an HPLC–MS/MS based enantioselective method for quantitation) was needed. Applications for enantioselective separation of some azole compounds by HPLC report the usage of polysaccharide-based chiral stationary-phases operated under normal or reverse phase conditions [15]. Among these, only the reverse-phase applications could potentially be coupled with MS/MS. In fact, in one scientific paper the separation of the imazalil enantiomers with reverse-phase chromatography was studied [16]. In this study, a column with underivatised β-cyclodextrin bonded to silica as a chiral selector (ChiraDex®) was eluted with isopropanol and phosphate buffer solution (1:99 v/v) (pH 4) [16]. This separation was suitable for

* Corresponding author.
E-mail addresses: kb@envs.au.dk, kb@dmu.dk (K. Bester).

http://dx.doi.org/10.1016/j.chroma.2016.05.008
0021-9673/

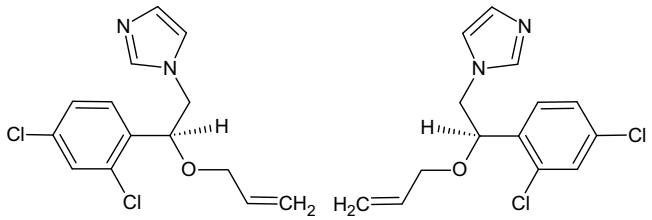

Fig. 1. Structures of imazalil enantiomers. (*S*)-Imazalil (Left) and (*R*)-Imazalil (Right) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

UV detection but would not be viable for MS due to the presence of the phosphate buffer. Alternatively, a manufacturer application indicated that imazalil could be separated by RP-HPLC using underivatized β-cyclodextrin as chiral selector, eluted with acetonitrile and 0.1% trimethylamine/acetate buffer (10:90 v/v) (pH 4.1) [17]. This option would be suitable for mass-spectrometry detection. In addition to these HPLC methods, the chiral resolution of imazalil was also performed by capillary electrophoresis (CE), again with β-cyclodextrin (derivatized) as a chiral selector [14]. To gain the required selectivity but also to gain robustness of the method, a new method based on derivatized β-cyclodextrin was designed.

Only a single study connects the absolute configuration of the imazalil enantiomers with optical rotation values [12]. The present study uses a combination of optical rotation and vibrational circular dichroism as well as electronic circular dichroism spectroscopy together with quantum chemical calculations to assign the absolute configuration of the imazalil enantiomers [18–24]. All three methods work in solution, an important advantage over X-ray diffraction which requires a single crystal of high quality.

Information on the absolute configuration was needed to conduct later toxicological studies on enantioselective endocrine-disrupting effects. Therefore, an HPLC-UV method was developed to be used for isolation of the enantiomers of imazalil. In addition, an HPLC–MS/MS method was designed for the enantioselective quantitation of imazalil enantiomers in cell medium samples from an *in vitro* toxicological test (H295R steroidogenesis assay [25]).

2. Materials and methods

2.1. Chemicals

Racemic imazalil was obtained from Fluka (Sigma Astec Inc., purity 99.8%). Gradient grade methanol was obtained from Merck, Darmstadt, Germany and water was LC–MS quality from Fluka (Sigma Aldrich, Steinheim, Germany).

The HPLC mobile phase additives were glacial acetic acid (AA) 100% anhydrous from Merck and triethylamine (TEA) (>99.5% purity) from Fluka. Dichloromethane (LiChroSolv, purity ≥ 99.9%) was obtained from Merck. Anhydrous sodium sulfate (purity was ≥ 99.0%) was obtained from Merck.

Tetrachloromethane for spectroscopy (CCl_4) (99+%) was purchased from Acros, Darmstadt, Germany, acetonitrile (MeCN) (HPLC grade) from Fisher Scientific, Nidderau, Germany and deuterochloroform ($CDCl_3$) (99.8%) from Deutero, Kastellaun, Germany.

2.2. Instruments and methods

2.2.1. Isolation of imazalil

An HPLC-UV method was developed for the isolation of enantiopure imazalil. The instrument used consisted of a dual low pressure mixing ternary gradient system (Dionex 3000 series, equipped with a 3000 TSL autosampler, column oven and degasser from the dionex 3000 series as well) coupled with a UV (DAD) detector from the Dionex 3000 series. The separation was conducted using a Nucleodex β-PM column from Macherey-Nagel, Düren, Germany (L: 200 mm, i.d.: 4 mm, 5 μm particles). It was operated with isocratic elution using 30% water (with 0.2% TEA, 0.08% AA at pH = 7) and 70% methanol at a flow of 0.7 mL/min (25 °C). Injections of 10 μL of a methanolic solution containing 20 mg/mL of racemic imazalil were performed and the separation was achieved within 7 min (Fig. 2). To obtain sufficient amounts of imazalil enantiomers, 100 injections with 9 min cycle time were conducted. During the injections, the imazalil enantiomers were collected separately in 100 mL flasks, utilizing the two Valco-valves of the system.

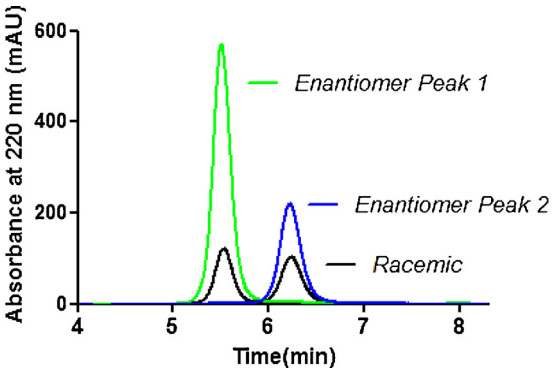

Fig. 2. Separation of imazalil enantiomers. Chromatogram of a racemic imazalil (black, Racemic) from a calibration standard (10 μL injection, 100 μg/mL) and chromatogram of the prepared fractions (10 μL injection) (enantiomer Peak 1, green and enantiomer Peak 2, blue), where each fraction contained one of the enantiomers. The retention times confirm that the isolation and further extraction of enantiomers was effective (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

The collected fractions were concentrated to approx. 5 mL on a rotary evaporator (water bath temperature 40 °C). The concentrates were diluted with 90 mL water from a Milli-Q system and extracted once with 20 mL CH_2Cl_2 and twice with 15 mL CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4 and evaporated to dryness. The enantiomeric purity was determined by enantioselective HPLC-UV.

2.2.2. Enantioselective determination of imazalil in samples from in vitro experiments

Isolated and purified imazalil enantiomers as well as racemic imazalil were used in an *in vitro* H295R steroidogenesis assay with the H295R human adrenocortical carcinoma cell line [25] for testing of the endocrine disrupting potential. Test conditions were applied as described in Sørensen et al. [26] with modifications. The medium consisted of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12) (GibcoBRL Life Technologies, Paisar, UK) and 10 mL/L of ITS + premix (BD Bio-science, Brøndby, Denmark).

The chromatographic method described above was adapted to HPLC–MS/MS for the analysis of imazalil enantiomers in cell medium samples. The instrument used also consisted of a dual low-pressure mixing ternary-gradient system Ultimate 3000 from Dionex, which had a pump of the 3000 series (DGP–3600 M), a WPS 3000 TSL autosampler and a column oven and degasser also from the Dionex 3000 series. The HPLC was coupled to an API 4000 mass spectrometer (ABSciex, Framingham, MA, USA). It was operated with an ESI source in positive mode set to 550 °C and 5500 V capillary voltage and operated using multireaction monitoring (MRM).

The enantioselective separation was transferred to HPLC–MS/MS by using the same analytical column and mobile phases as in the HPLC-UV method (see Section 2.2.1). The following step-gradient was used: water/methanol 100:0 (0 min–3 min) 100:0 → 30:70 (3 min–4 min) 30:70 (4 min–18 min) 30:70 → 0:100 (18 min–19 min) 0:100 (19 min–20 min) 0:100 → 100:0 (20 min–