International Journal of Environmental Analytical Chemistry

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/geac20>

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Jin-Hui Chen^a, Hui-Li Wang^a, Bao-Yuan Guo^a & Jian-Zhong Li^a

^a Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

Published online: 20 Nov 2013.

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To cite this article: Jin-Hui Chen, Hui-Li Wang, Bao-Yuan Guo & Jian-Zhong Li (2014) LC-MS/MS method for simultaneous determination of myclobutanil, hexaconazole, diniconazole, epoxiconazole and tetraconazole enantiomers in soil and earthworms, International Journal of Environmental Analytical Chemistry, 94:8, 791-800, DOI: [10.1080/03067319.2013.853763](https://doi.org/10.1080/03067319.2013.853763)

To link to this article: <http://dx.doi.org/10.1080/03067319.2013.853763>

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LC-MS/MS method for simultaneous determination of myclobutanil, hexaconazole, diniconazole, epoxiconazole and tetraconazole enantiomers in soil and earthworms

Jin-Hui Chen, Hui-Li Wang, Bao-Yuan Guo* and Jian-Zhong Li

Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

(Received 13 May 2013; final version accepted 25 August 2013)

A new method using gel permeation chromatography (GPC) clean-up followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed for the determination of myclobutanil, hexaconazole, diniconazole, epoxiconazole and tetraconazole enantiomers in soil and earthworms. The soil and earthworm samples were extracted with acetonitrile and then purified with a GPC procedure conducted on a CO785 column with cyclohexane and ethyl acetate (50:50, V/V) as the mobile phase at a flow rate of 4.7 mL/min. The eluent between 7.5 min and 15 min was collected for LC-MS/MS analysis. The LC mobile phase and MS/MS conditions were optimised. Formic acid (0.1%, V/V) was added to the water phase to enhance MS responses. The LODs of the enantiomers were between 0.001–0.003 mg/kg. At three fortification levels of 0.01–1.00 mg/kg, recoveries were between 81.2% and 100.2% in soil and earthworms. The relative standard deviation was below 14%. The results of earthworm experiment showed that hexaconazole and diniconazole might have the bioaccumulation potential, whereas tetraconazole, epoxiconazole and myclobutanil were most likely to undergo enantioselective biotransformation. On the contrary, the degradation of the triazoles in soil was achiral, and less than 10% of them were degraded after 21 days.

Keywords: bioaccumulation; chiral separation; LC-MS/MS; triazole fungicides

1. Introduction

Every year, thousands of tons of pesticides are released into the environment, which cause pollution and ecological risk. More than 30% of these pesticides are chiral with at least one pair of enantiomers [1–3], but the differences between the enantiomers are usually ignored in regulation and practice [4]. Triazoles are a class of the most commonly used chiral pesticides with characteristics of hydrophobicity and persistence, which take effect by inhibiting cytochrome (CYP) P-450 [5,6]. The enantiomers of each triazole often exhibit significantly different behaviour. For instance, (–)-diniconazole is more fungitoxic than (+)-diniconazole [7]; (+)-tebuconazole degrades faster than (–)-enantiomer in cabbage, rat liver microsome and rabbit plasma, whereas (–)-tebuconazole dissipates faster than the (+)-tebuconazole in cucumber and soil [8–10]. Previous studies also showed that (+)-tebuconazole was accumulated from soil to earthworms with a biota to soil accumulation factor (BASF) of 1.64, whereas (–)-tebuconazole did not accumulate in earthworms [11].

Several studies have focused on the determination of triazoles in soil [12–15]. Furthermore, a number of technologies, such as high-performance liquid chromatography (HPLC) [16–20],

*Corresponding author. Email: guoby@rcees.ac.cn

Figure 1. The structure of myclobutanil, hexaconazole, diniconazole, epoxiconazole and tetraconazole. (Chiral centre at carbon noted by an asterisk (*) in the structures).

gas chromatography, capillary electrophoresis (CE) [21–23], supercritical fluid chromatography (SFC) [24], thin layer chromatography (TLC), simulated moving bed chromatography (SMBC), countercurrent chromatography and microtechnology have been introduced in enantiomers' analysis [25,26]. In recent years, HPLC-tandem mass spectrometry (HPLC-MS/MS) has shown huge advantages in pesticides analysis for its sensitivity and selectivity. However, the application to enantiomer's analysis is scarce. The most important reason is that the normal phase is unsuitable for HPLC-MS/MS under most conditions.

In the present study, a reverse phase HPLC-MS/MS method was developed to determine ten enantiomers of five triazole fungicides (myclobutanil, hexaconazole, diniconazole, epoxiconazole and tetraconazole) (Figure 1) in soil and earthworms. This method was employed to investigate the bioaccumulation and biotransformation of the triazole fungicides by dietary exposure to earthworm.

2. Experimental

2.1 Reagents and materials

Methanol, acetonitrile, formic acid, cyclohexane and ethyl acetate were HPLC grade from Merck (Darmstadt, Germany). High-purity water made by Milli-Q (Millipore, Bedford, MA) was used during the whole analysis. All other chemicals were analytical reagent grade from Beijing Chemical Factory Company (Beijing, China).

Myclobutanil, hexaconazole, diniconazole, epoxiconazole and tetraconazole were purchased from Dikma (Beijing, China) with purity of 99.5 % all. Individual stock standard solutions were established by dissolving the pesticides in acetonitrile at a concentration of 1,000 mg/L. Work solutions of myclobutanil, hexaconazole, diniconazole, epoxiconazole were prepared in acetone at a concentration of 250 mg/L, and of 100 mg/L in the case of tetraconazole. All solutions were stored in a refrigerator at 4 °C before use.

Soil was collected from Chaoyang District, Beijing, China. And it was air-dried at room temperature, homogenised, and passed through a 2 mm sieve before use. The soil was a sandy loam soil (78% sand; 7% silt and 15% clay) with 4.7% organic matter. The soil pH was 6.8. Earthworms were obtained from the Institute for the Control of Agrochemicals, Ministry of

Agriculture (ICAMA). All materials were examined before use, to ensure they were free of target analytes.

2.2 Experiment

Five hundred grams of dry soil were weighed and thoroughly mixed with 100 mL of the work solutions and then air-dried in a fume hood for 24 hours. After solvent evaporation, deionised water was added to adjust the moisture content to 40–60%. The treated soil was put into a 1 L glass beaker and incubated for 4 days. 24 replicates of each fungicide and three controls were prepared, respectively. Ten earthworms with similar weight (0.3–0.6 g) were put in every beaker that were kept in man-made climate equipment setting the temperature at 20 ± 2 °C with a photoperiod of 16:8 hours light/dark cycle. The food ratio and soil moisture were controlled by re-weighing the test vessels and evaporated water was compensated every 7 days. Three replicates of soil and worm samples were taken after 0.25, 1, 2, 4, 7, 11, 14 and 21 days.

2.3 Sample pretreatment

One gram of soil or earthworm sample was weight into a flask. Ten grams of anhydrous sodium sulfate and 20 mL acetonitrile were added. The mixture was homogenised with a T-25 homogeniser (IKA, Deutschland, Germany) and then exposed to an ultrasonic bath for 10 min, followed by centrifugation at 3000 r/min for 5 min. The organic layer was filtered through anhydrous sodium sulfate (10 g) and evaporated to dryness on an R-215 Rotavapor (Buchi, Flawil, Switzerland) at 30 °C. The residue was dissolved by 10 mL of a mixture of cyclohexane and ethyl acetate (50:50, V/V) for further clean-up.

The clean-up procedure was performed 7.5 mL of the above solution on an AccuPrep MPS & AccuVap Inline Gel Permeation Chromatography (GPC) system (J2 Scientific, Columbia, USA) coupled with a CO785 column (Bio-beads S-X3). A mixture of cyclohexane and ethyl acetate (50:50, V/V) was used as mobile phase, and the flow rate was set to 4.7 mL/min. The eluent was monitored with a UV detector (at 254 nm) and was collected between 7.5–15 min for further concentrating in the AccuVap Inline system. At the endpoint, the residues were dissolved in 2 mL of acetonitrile, and diluted 10 to 100 times before injection into the HPLC-MS/MS system.

2.4 HPLC-MS/MS analysis

LC was performed on a Waters Acquity UPLC system (Waters Corp., Mildford, MA) equipped with a binary solvent manager system. Three chiral stationary phase (CSP) columns were tested in this study. CHIRALPAK IB [cellulose tris(3,5-dimethylphenyl carbamate)] was an immobilised CSP column with 150mm long, 4.6mm (i.d.) and 3 µm diameter; CHIRALCEL OD-3R [cellulose tris (3,5-dimethylphenyl carbamate)], and CHIRALPAK AD-3R [amyasetris (3,5-dimethylphenyl carbamate)] both were 150 mm long, 4.6 mm (i.d.) with different enantioselective phases coated onto 3 µm silica gel beads. All the columns were purchased from Daicel (Shanghai, China).

The mobile phase was solvent A (0.05% formic acid in ultrapure water) and solvent B (acetonitrile) in 40:60 V/V ratio and at 0.25 mL/min. The injected volume was 5 µL. The column was kept at 30°C and the temperature in the sample manager was kept at 10 °C. Mass spectrometry was performed on a Quattro Premier XE Mass Spectrometer (Micromass, UK). The desolvation gas (nitrogen) flow was set to 600 L/h at a temperature of 350 °C; the cone gas (nitrogen) was set to 50 L/h, and the electrospray ionisation (ESI) source temperature to 120 °C. The capillary and cone voltages were set to 1.7 kv and 30 V, respectively. The Masslynx NT

Table 1. The resolution of the five fungicides on column CHIRALCEL OD-3R, CHIRALPAK AD-3R and CHIRALPAK IB-3.

Compound	OD-3R	AD-3R	IB-3
Myclobutanil	3.19	1.02	1.32
Hexaconazole	1.62	0.45	–
Diniconazole	1.51	0.30	–
Epoxiconazole	10.96	–	1.97
Tetraconazole	2.26	–	1.00

v.4.1 (Waters) software was used to control the instrument, and analyse the data obtained. The Electrospray ionisation (ESI) source was operated in positive mode. MS analyses were performed in the multiple reaction monitoring (MRM) modes as shown in Table 1.

3. Results and discussions

3.1 Optimisation of GPC clean-up method

GPC was an effective clean-up procedure in the separation of synthetic macromolecules. The enzymes, protein, fat and other macromolecular materials in earthworms and soil could easily be clean-up by this method. Therefore, GPC was selected for the clean-up of soil and earthworm samples.

Before running samples, the elution curve was studied. Mixed standard solutions and control samples were injected into the system. The GPC chromatogram showed that the five fungicides were eluted between 7.5 to 12.5 min, both in the standard solution (Figure 2A) and the soil and earthworm samples (Figure 2B). The GPC recoveries of the five fungicides were above 90% all.

3.2 HPLC-MS/MS condition optimisation

3.2.1 Column selection

The selection of column was important in the separation of chiral pesticides. Polysaccharide chiral stationary phases (CSP) account for approximately a third of all HPLC chiral separations [27]. For this study, three CSP chiral columns, CHIRALPAK IB-3, CHIRALCEL OD-3R and CHIRALPAK AD-3R, were tested. Although the epoxiconazole molecule contain two chiral carbons (Figure 1) and four stereoisomers, the present commercial product just contains a pair of enantiomers with 2R, 3S- and 2S, 3R- configuration [28,29]. Thus, there were ten enantiomers to be separated in this study.

Figure 2. A: Elution chromatogram of myclobutanil, hexaconazole, diniconazole, epoxiconazole and tetraconazole, B: Elution chromatogram of earthworm control, mixed standard and soil.

Column CHIRALCEL OD-3R is the 3 μm silica support reverse phase version of CHIRALCEL OD. Column CHIRALPAK IB-3 is the 3 μm silica support version of CHIRALPAK IB. CHIRALCEL OD-3R is a coated column, whereas the CHIRALPAK IB-3 is an immobilised column. Although the CHIRALPAK IB is considered as the immobilised version of CHIRALCEL OD, more proportion of the racemates were separated on CHIRALCEL OD than on CHIRALPAK IB [30]. In this study, the results showed that only myclobutanil, epoxiconazole and tetraconazole became separated on column CHIRALPAK IB-3. Besides, we observed higher separation factors in CHIRALCEL OD-3R. In Table 1 it was shown that the column types played an important role in the enantiomer separations. The column CHIRALCEL OD-3R exhibited greatest enantiomer recognition in the three columns, so this column was selected in this study.

3.2.2 Mobile phase optimisation

Methanol and acetonitrile, as the two most frequently used mobile reverse-phases, were investigated. A satisfactory enantioselective separation of the ten enantiomers was achieved when acetonitrile was used.

Formic acid was added in water phase to enhance the ionisation for higher sensitivity. Different concentrations (0, 0.05%, 0.1%, 0.2%, V/V) were investigated. Compared to total water phase, the responses of fungicides were all enhanced by low concentration of formic acid. Hexaconazole and myclobutanil were greatly affected by the changing of the formic acid concentrations. The responses of the two fungicides were reduced more than 50% when the concentrations of formic acid increased from 0.05% to 0.2%. Tetraconazole and diniconazole had the largest responses when the formic acid concentration was 0.1% in water. But the response of diniconazole did not changed more than 10% within a formic acid concentration range from 0.05% to 0.1%. The situation of epoxiconazole was similar to diniconazole, it was not sensitive to the formic acid concentration. The responses neither changed significantly for hexaconazole and myclobutanil. The final mobile phase was set to A (0.05% formic acid in ultrapure water): B (acetonitrile) = 40: 60 (V/V), and the flow rate was at 0.25 mL/min.

3.2.3 MS/MS condition

The MS and MS/MS tuning procedures were done one chemical by one chemical to optimise the MS/MS condition. The primary multiple reactions monitoring (MRM) method for each analyte was incorporated into a method for the five fungicides. In total, ten MRM transitions were monitored in one time window, which contained the information obtained during the instrument tuning process (Table 2).

In the process of tuning, it was found that the sensitivity varied by different tune files. The most different was in capillary voltage (Table 2). The relationship between capillary voltage and spectrum areas were studied, but no functional relationship was found. The only finding was

Table 2. The analytes fragmentation and instrumental conditions.

Compound	Parent (Da)	Cone (V)	Product ion (Da)	Collision (eV)	Capillary (V)
Myclobutanil	289.07	35.00	70.10, 125.07	16.00, 32.00	1.60
Hexaconazole	314.06	34.00	70.00, 159.00	30.00, 28.00	2.00
Diniconazole	326.17	35.00	70.19, 159.13	24.00, 36.00	1.70
Epoxiconazole	330.16	30.00	101.15, 121.11	42.00, 16.00	1.10
Tetraconazole	371.85	40.00	70.00, 158.97	20.00, 34.00	3.50

that the peak areas were larger than others when the capillary voltage was set as the diniconazole tune file, so the diniconazole tune file was used in this method.

3.3 Method validation

Method validation was evaluated by the following parameters of specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision. Blank samples (soil, earthworm) were analysed to verify the absence of interfering species at the retention time of the analyte. The elution order of triazole fungicides is shown in Figure 3.

Figure 3. The separation of myclobutanil, hexaconazole, diniconazole, epoxiconazole and tetraconazole on CHIRALCEL OD-3R. (mobile phase: A (0.05% formic acid in ultrapure water): B (acetonitrile) = 40:60, flow rate 0.25 mL/min).

Table 3. The determination of linearity, LOD and LOQ of each enantiomer.

Compound	Regression equation	LOD (mg/kg)	LOQ (mg/kg)	R ²
(+)-tetraconazole	y = 20194x – 138.3	0.001	0.003	0.9996
(-)-tetraconazole	y = 20246x – 5.237	0.001	0.003	0.9987
(+)-hexaconazole	y = 9203.x + 259.0	0.003	0.01	0.9992
(-)-hexaconazole	y = 9202.x + 261.1	0.003	0.01	0.9971
(-)-cis-epoxiconazole	y = 9488.x + 112.3	0.002	0.005	0.9989
(+)-cis-epoxiconazole	y = 9611.x – 180.8	0.002	0.005	0.9992
(+)-myclobutanil	y = 10343x – 79.03	0.001	0.003	0.9994
(-)-myclobutanil	y = 11582x – 367.0	0.001	0.003	0.9986
(-)-diniconazole	y = 8060x – 27.96	0.002	0.005	0.9985
(+)-diniconazole	y = 8627.x – 100.3	0.002	0.005	0.9981

The linearity of this method was determined by analysing the standard solutions in triplicate at five concentrations, ranging from 0.01 to 1.00 mg/L. All linearities were satisfactory (Table 3), being the correlation coefficient (R^2) higher than 0.99. The LODs and LOQs of the method were determined by spiking standards in blank samples of soil and earthworm. The LODs for two enantiomers of each fungicide were the concentration that produces a signal-to-noise (S/N) ratio of 3, whereas the LOQs were defined based on an S/N ratio of 10. Accuracy and precision of the method were investigated by recovery assays. In accordance with specific conditions of this study, five replicates of spiked samples at three levels (0.01 mg/kg, 0.10 mg/kg and 1.00 mg/kg) were prepared. The recoveries were between 81.2% and 100.2% in soil and earthworms. The relative standard deviation was below 14% for each enantiomer.

3.4 Analysis of real samples

The method described above was applied to study the enantioselective accumulation of the five fungicides in earthworms. The earthworms were exposed to the five racemic triazole fungicides under the concentration of 25 mg/kg (dry weight) for hexaconazole, epoxiconazole, myclobutanil, diniconazole, and 10 mg/kg (dry weight) for tetraconazole in soil. After 21 days of culture, the concentrations of the enantiomers were almost equal in soil. The degradations were below 10%. Enantioselective accumulations were found in earthworm as shown in Figure 4. As it can be seen, the steady state occurred when the plot of the concentration in worms against time was parallel to the time axis, and three successive concentration analyses made on samples taken at intervals of at least 2 days do not vary more than $\pm 20\%$ of each other, based on statistical comparisons (e.g., analysis of variance, regression analysis). The accumulations of the fungicides reached a plateau in about 11 days. The steady state bioaccumulation factors (BAF_{ss}), calculated by the ratio of the concentration in worms (C_a) and in the soil (C_s) at the apparent steady state, was used to evaluate the accumulation of the fungicides. The BAF_{ss} were 5.94 for (+)-hexaconazole and 5.48 for (-)-hexaconazole, 4.94 for (+)-diniconazole and 5.48 for (-)-diniconazole, 3.33 for (+)-tetraconazole and 3.20 for (-)-tetraconazole, 1.48 for (+)-epoxiconazole and 2.00 for (-)-epoxiconazole, 1.05 for (+)-myclobutanil and 1.22 for (-)-myclobutanil (Table 4).

The enantiomer fraction (EF) was employed to express the enantioselectivity, which was calculated by the equation:

$$EF = A_{(+)} / (A_{(+)} + A_{(-)})$$

Figure 4. Concentrations of each enantiomer in earthworm and soil samples. The start concentration of each enantiomer of myclobutanil, hexaconazole, diniconazole, epoxiconazole was 25 mg/kg (dry weight) in soil. The start concentration of each enantiomer of tetraconazole was 10 mg/kg (dry weight) in soil.

Table 4. The steady state bioaccumulation factor (BAF_{ss}) of the five fungicides in earthworm.

Compound	BAF_{ss}	Compound	BAF_{ss}
(+)-Hexaconazole	5.94	(-)-Hexaconazole	5.48
(-)-Diniconazole	5.48	(+)-Diniconazole	4.94
(+)-Tetraconazole	3.33	(-)-Tetraconazole	3.20
(-)-Epoxiconazole	2.00	(+)-Epoxiconazole	1.48
(-)-Myclobutanil	1.22	(+)-Myclobutanil	1.05

where A₍₊₎ is the peak area of (+)-enantiomer, and A₍₋₎ is that of (-)-enantiomer. The EF values range from 0 to 1, with EF = 0.50 indicating a racemic mixture.

In this study, the EF of hexaconazole increased from 0.5 (initial time) to 0.52 (ending time), whereas the EF of tetraconazole, 0.5 (initial time) to, 0.51 (ending time) in the period of experiment. However, the EF of diniconazole decreased from 0.5 (initial time) to 0.47 (ending time); the EF of epoxiconazole decreased from 0.47 (initial time) to 0.43 (ending time); the EF of myclobutanil decreased from 0.49 (initial time) to 0.48 (ending time). So, the (+)-hexaconazole and (+)-tetraconazole were more likely to bioaccumulate than their (-)-enantiomers, whereas the (-)-diniconazole, (-)-epoxiconazole and (-)-myclobutanil were more likely to bioaccumulate than their (+)-enantiomers.

4. Conclusions

In this study, A GPC clean-up coupled with reverse phase HPLC-MS/MS for simultaneous quantitative determination of five triazole fungicide enantiomers in soil and earthworms was developed. The method was successfully validated. All data indicated that HPLC-MS/MS was suitable to determine myclobutanil, hexaconazole, diniconazole, epoxiconazole and tetraconazole in soil and earthworms. The 21 days of culture experiment showed that the bioaccumulation in earthworm reached a steady state after 11 days. Besides, hexaconazole and diniconazole showed high risk of bioaccumulation, and tetraconazole, epoxiconazole and myclobutanil showed enantioselective biotransformation in the study.

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