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Enantiomeric Separation and Circular Dichroism Detection of Metalaxyl Acid Metabolite by Chiral High Performance Liquid Chromatography

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The enantiomers of metalaxyl acid metabolite (MX-acid) were separated by HPLC using cellulose-*tris*-(3,5-dimethylphenyl-carbamate) (CDMPC) as chiral stationary phase. The effects of mobile phase composition, flow rate and column temperature on the resolution were investigated and good separation was achieved with *n*-hexane/2-propanol/trifluoro-acetic acid as mobile phase. The circular dichroisms detection showed that metalaxyl-acid has similar absorption behaviour to metalaxyl with CD signals reverse to optical rotation direction of S(+)- and R(-)-enantiomer and CD signals of metalaxyl intermediate enantiomers may be reversed with change of CD wavelength.

Key Words: Metalaxyl, Metabolite, Enantiomeric separation, Circular dichroism.

INTRODUCTION

Metalaxyl (MX, Fig. 1) is a chiral acetamide fungicide and synthesized by intermediate (2-(2,6-dimethyl-phenylamino) propionic acid methyl ester, MX-inter). It is widely used for control of plant diseases caused by *Phythophthora infestans* and *Pythium ultimum*. Metalaxyl consists of a pair of enantiomers, previous study demonstrated that its fungicidal activity almost entirely come from the R(-)-enantiomer[1]. So in recent years, racemic metalaxyl has been replaced by metalaxyl-M (MX-M) containing a minimum of 97 % of the active R(-)-enantiomer.

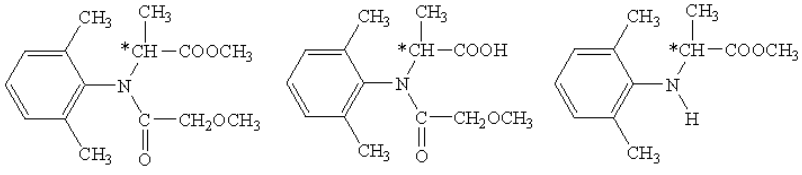

Fig. 1. Chemical structures of MX, MX-acid and MX-inter.

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Metalaxyl acid (2-(2-methoxy-N-(2,6-dimethylphenyl)acetamido)propanoic acid, MX-acid) is a main metabolism/degradation product of MX in animal/soil[2]. MX-acid is also a chiral compound and has two enantiomers. Traditionally, MX-acid enantiomers were separated and analyzed by gas chromatography or gas chromatography-mass spectrometry (GC-MS) after derivatization. For example, MX-acid enantiomers extracted from soil sample were first derivatized using diazoethane to form corresponding ethyl ester and allow distinction of MX (a methyl ester) and then detected by enantioselective GC-MS[3]. However, its chromatographic behaviours on chiral column and circular dichroism have not been reported. At present, two main chiroptical properties-based detector are the optical rotation detector (OR) and circular dichroism detector (CD). Optical rotation detector is based on difference of refractive index between left and right linearly polarized lights and thus highly affected by changes of temperature and solvent. Circular dichroism detector is based on difference of absorption between left and right circularly polarized light and intrinsically steady during variety of temperature and solvent. Comparatively, UV is about 10 times more sensitive than CD and about 100 times than OR[4]. Because of only responding to the nonracemic optical molecule and thus can get a clean chromatogram to analyze, in recent years CD get more and more application in qualitative, quantitative and absolute configuration determination of chiral compounds especially in complex sample[5,6].

Present study is aim to understand chromatographic behaviours and circular dichroism of metalaxyl-acid. Firstly, metalaxyl-acid was prepared by hydrolyzing of metalaxyl. Then the effects of 2-propanol content, flow rate and column temperature on separation of metalaxyl-acid enantiomers on cellulose-*tris*-(3,5-dimethylphenylcarbamate) (CDMPC) chiral stationary phase (CSP) were investigated. Finally, circular dichroisms of MX, MX-acid and MX-intermediate were comparatively detected.

EXPERIMENTAL

Racemic MX, MX-intermediate and MX-M were obtained from Lianyungang Liben Agro-chemical Co., Ltd (purity >97 %) (Jiangsu Province, China). 2-propanol (IPA) and *n*-hexane (HPLC grade) were purchased from Beijing Chemical Reagents Company (Beijing, China) and filtered through 0.45 μm filter membrane before use. Trifluoroacetic acid was purchased from Sigma-Aldrich. All other chemicals and solvents were analytical grade and purchased from commercial sources. MX-acid was obtained by hydrolysis of MX according to reference and qualitatively confirmed by Bruker 300 MHz NMR and Agilent 5973 Inert MS[7].

Chromatograhpy: An Agilent 1100 series HPLC was used to chromatographic separations, equipped with a G1322A degasser, G1311A quatpump, G1316A column compartment, G1315B diode array detector (DAD), G1328B manual injector and a 20 μL sample loop (Wilmington, DE, USA). The scanning wavelength of DAD was set from 190 to 400 nm. The signal was acquired and processed by an Agilent Chemstation for LC 3D.

A Jasco 2000 HPLC system was used to detect circular dichroism (Jasco, Tokyo, Japan), consisted of a PU-2089 plus pump, CD-2095 plus circular dichroism (CD) detector, Reodyne model 7725 injector with 20 μL sample loop and a Chrompass workstation. The scan range of CD wavelength was between 220 and 400 nm.

Enantiomeric separations were achieved using cellulose-*tris*-(3,5-dimethylphenyl-carbamate) coated on aminopropylsilanized spherical silica gel as CSP and *n*-hexane/2-propanol as mobile phase. The CSP was synthesized and packed into a 250 × 4.6 mm (i.d.) stainless steel column[8]. The void time (t_0) was determined by 1,3,5-tri-*tert*-butylbenzene. Chromatographic parameters were calculated using follow equations: resolution factor (Rs) = 2 $(t_2-t_1)/(W_2+W_1)$, W_1 and W_2 were peak width of first and second eluting enantiomers, respectively;capacity factor (k) = $(t_R-t_0)/t_0$; separation factor (α) = k_2/k_1, k_1 and k_2 were capacity factors of first and second eluting enantiomers, respectively.

RESULTS AND DISCUSSION

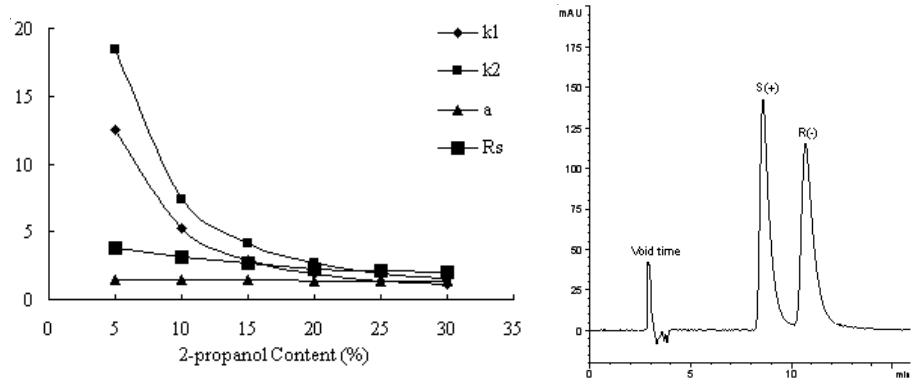
Effects of 2-propanol content on separation: Metalaxyl-acid can't be eluted from CSP even if using 100 % of 2-propanol because of strong hydrogen bond interaction between carboxyl of metalaxyl-acid and hydroxyl of CSP. So 0.1 % of trifluoroacetic acid was added in mobile phase to inhibit this interaction and accelerate enantiomeric elution. Fig. 2a showed effects of 2-propanol content in mobile phase on chromatographic separation of metalaxyl-acid enantiomers (flow rate was 1.0 mL/min, column temperature was 25 °C). When 2-propanol content increased from 5 to 30 %, capacity factor (k) decreased as expected, separation factor (α) and resolution factor (RS) also decreased but resolution factor was more apparent than α. Resolution factor descended from 3.76 to 1.96, the reason may be influences of 2-propanol on interaction between CDMPC and two enantiomers were different. Comparatively, the separation effect of metalaxyl enantiomers on CDMPC was apparently better than that of metalaxyl-acid with resolution factor of 5.32 at 15% of 2-propanol content[9], which indicates that methyl ester cleavage of metalaxyl significantly affect its discrimination and interaction with CDMPC.

Effects of flow rate on separation: When the flow rate increased from 0.5 to 2.5 mL/min (column temperature was 25 °C, *n*-hexane/IPA/TFA was 80/20/0.1 %), Rs decreased from 2.22 to 1.78, which may be caused by accelerating elution of enantiomers from CSP and leading to short interaction time between the analyte and CDMPC. But k had little variety with k_1 changing from 2.136 to 2.066 and α nearly had no change with flow rate increasing, which means that relative retention of two enantiomers on CDMPC was invariable under this condition.

Effects of column temperature on separation: When column temperature increased from 0 to 40 °C (flow rate was 1.0 mL/min, *n*-hexane/IPA/ TFA was 80/20/0.1 %), k apparently reduced with k_1 decreasing from 3.284 to 1.72 but α nearly had no change, which indicates that relative retention of two enantiomers on CDMPC was invariable with column temperature changing. Resolution factor had minor

fluctuation and the best resolution was obtained at 10 °C with resolution factor of 2.15. These results showed that column temperature has not obvious effect on enantiomeric separation.

According to above results, CDMPC showed excellent enantiomeric discrimination ability for two enantiomers of metalaxyl-acid. The optimized chromatogram of separation was shown in Fig. 2b. The S(+)-enantiomer was firstly eluted under this condition. This result was also confirmed by Buser[10] and following CD detection.



(a) (b)

Fig. 2. Effects of 2-propanol content on separation (a) and optimized chromatogram (b) of MX-acid (*n*-hexane/IPA/TFA = 80/20/0.1, 1.0 mL/min, 220 nm, 25 °C)

Circular dichroism detection of MX, MX-acid and MX-inter: S(+)-MX was firstly eluted on CDMPC with hexane/IPA as mobile phase[11]. CD and UV spectra of MX enantiomers and chromatograms of *rac*-MX and MX-M were shown in Fig. 3. CD spectrums of two enantiomers were approximately opposite to each other along wavelength, the S(+)- and R(-) -enantiomer showed negative (-) and positive (+) -absorption signal from 220 to 260 nm, respectively. The maximum CD-absorption wavelength of two enantiomers is 230 nm and no absorbance after 260 nm. It is worth to note that CD signals is reverse to optical rotation direction of S(+)- and R(-)-MX, which is not same to that lorazepam enantiomers have a consistent CD signal with optical rotation direction reported by Kanazawa etc., thereinto S(+) and R(-)-lorazepam showed CD (+) and CD (-) signal, respectively[12].

The elution order of MX-acid enantiomers on CDMPC had been reported by Buser with S(+)-enantiomer firstly eluted[10] and the result was same to that of MX. CD and UV spectra and chromatograms of MX-acid enantiomers were shown in Fig. 4. CD spectrums of two enantiomers were approximately opposite to each other along wavelength and the first and second eluted enantiomers showed (-)- and (+)-absorption signal from 220 to 260 nm, respectively. The maximum CD-absorption wavelength of two enantiomers is 230 nm and no absorbance after 260 nm. These results showed that MX-acid has similar CD characters with MX and methyl ester cleavage of MX has not significant effect for its CD absorption.

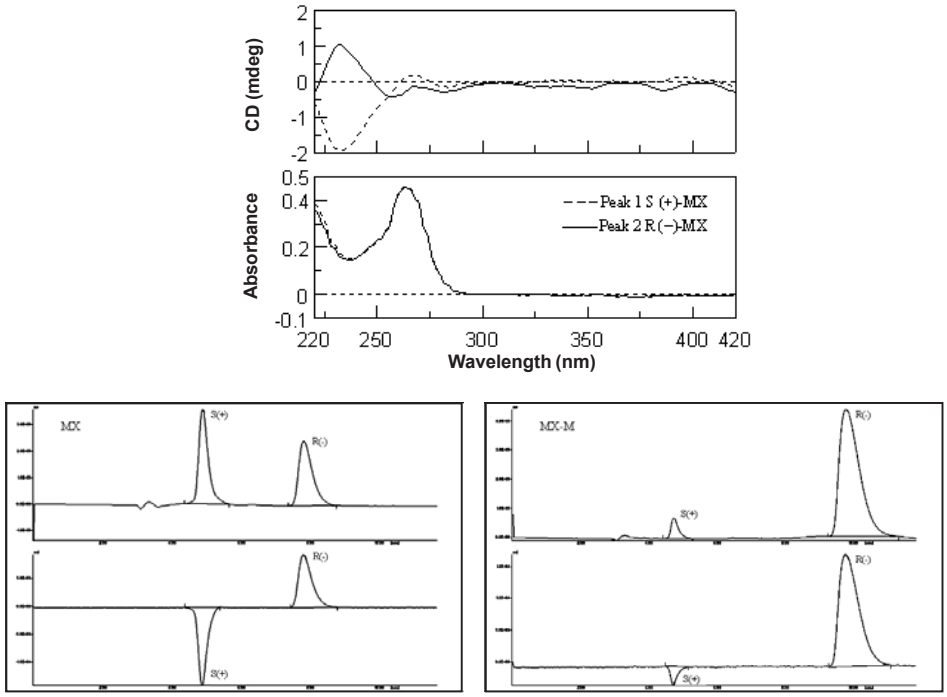


Fig. 3. CD and UV spectrums of MX enantiomers and chromatograms of *rac*-MX and MX-M at 230 nm, CDMPC column (250 × 4.6 mm), 1.0 mL/min, *n*-hexane/IPA = 60/40

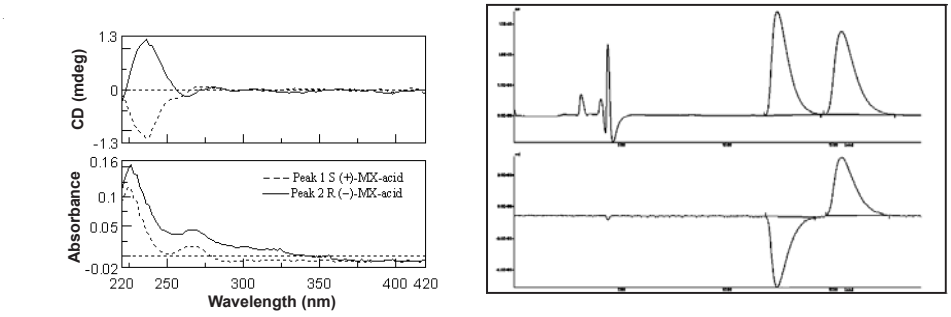


Fig. 4. CD and UV spectrums of MX-acid enantiomers and chromatograms at 230 nm, CDMPC column (250 × 4.6 mm), 1.0 mL/min, *n*-hexane/IPA/TFA = 85/15/0.1 %

The elution order of MX-inter enantiomers on CDMPC has not been reported. CD and UV spectra and chromatograms of MX-inter enantiomers were shown in Fig. 5. CD spectrums of two enantiomers were approximately opposite to each other along wavelength and the first eluted enantiomer showed (-)-absorption signal from 220 to 235 nm and (+)-signal from 235 to 305 nm. The maximum CD-absorption wavelength of two enantiomers is 250 nm and no absorbance after 305 nm. These

results are entirely different from those of MX and MX-acid enantiomers. UV and CD chromatograms of *rac*-MX-inter at 228 and 280 nm in Fig. 5 showed that the absorption signals of MX-inter enantiomers are opposite to each other at two detection wavelengths. This indicates the sequence of CD signal of enantiomers may be reversed with change of CD wavelength. Its reason was thought for that the monitoring of wavelength was selected outside the region where the π-π* transition of the arylketone occurs[13].

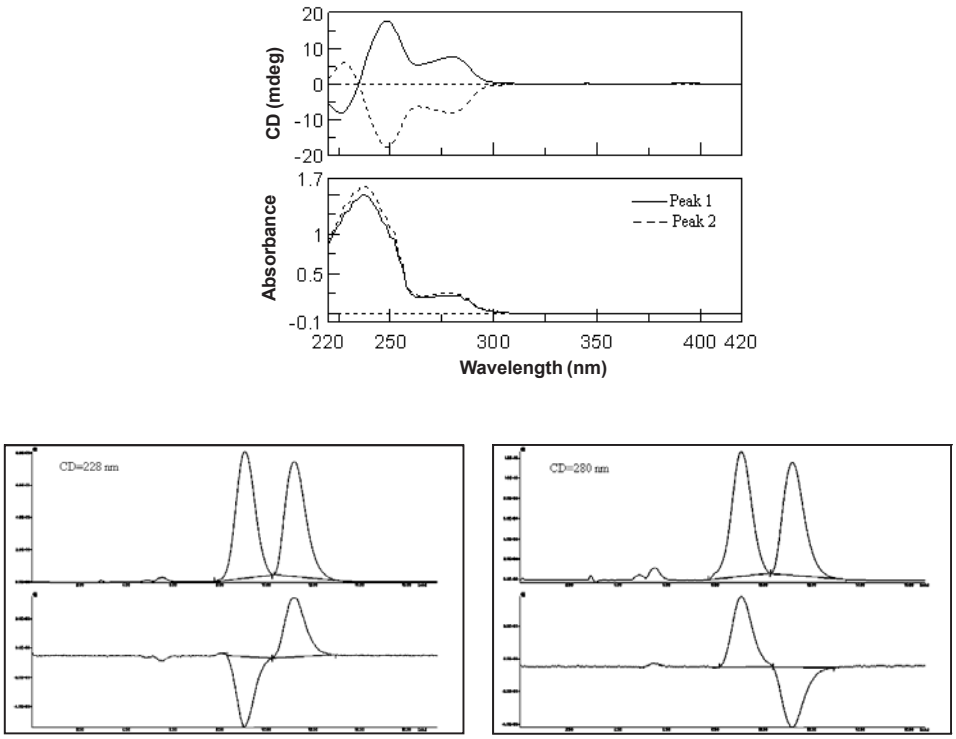


Fig. 5. UV and CD spectrums of racemic MX-inter and chromatograms at 228 nm and 280 nm, CDMPC column (250 × 4.6 mm), 1.0 mL/min, *n*-hexane/IPA=99.5/0.5

Conclusion

The chiral separation of MX-acid on CDMPC was investigated in this study. Two enantiomers of MX-acid can get good resolution but need add strong acid in mobile phase to restrain strong hydrogen bond interaction between carboxyl of MX-acid and hydroxyl of CSP. The enantiomeric CD detection of MX-acid, MX-inter and MX showed that MX-acid has similar CD spectrum with MX and CD signal of MX-inter enantiomers may be reversed with change of CD wavelength in two different absorption scopes.

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