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Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 97–107
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Separation and characterization of synthetic impurities of triclabendazole by reversed-phase high-performance liquid chromatography/electrospray ionization mass spectrometry

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Received 22 April 2004; received in revised form 28 September 2004; accepted 29 September 2004
Available online 23 November 2004

Abstract

A simple high-performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS) method for the separation and characterization of impurities in the synthesis of triclabendazole has been developed. The analytical separation was achieved on a reversed-phase C18 column using acetonitrile and water (60:40, v/v) as mobile solvent at a flow-rate of 1.0 ml/min at 25 °C, and an UV detection at 230 nm. The on-line HPLC/ESI-MS^n examinations were performed using ion trap analyzer with extraction ion chromatography (EIC) technique in positive or negative ion modes. The semi-preparative separation was performed with a reversed-phase column using methanol and water (75:25, v/v) as mobile solvent at a flow-rate of 4 ml/min at 25 °C, and an UV detection at 230 nm. Thus, two impurities were detected and identified as 5-chloro-6-(2,3,4-trichlorophenoxy)-2-methylsulfanyl-1*H*-benzoimidazole and 5-chloro-6-(2,3-dichlorophenoxy)-1-methyl-2-methylsulfanyl-1*H*-benzoimidazole. Meanwhile, some intermediates of impurity-1 in multi-step synthetic reactions, were tracked. Structural elucidation by 1D and 2D NMR and ESI-MS^n was discussed.

Keywords: Triclabendazole; Characterization; Reversed-phase HPLC; ESI-MS; Impurities; Structure elucidation

1. Introduction

Triclabendazole is a benzoimidazole anthelmintic widely used for the control of fasciolosis in ruminants [1]. It is highly effective against immature and adult stages of mature and immature *Fasciola hepatica* and *Fasciola gigantica* in small and large domestic ruminants [2]. It is also frequent treatments within the prepatent period can reduce the fluke infection to a negligible level [3–8].

The analysis of triclabendazole bulk drug revealed the presence of two impurities which were up to 0.1%. As per the stringent regulatory requirements the impurity profile study has to be carryout for any final product to identify and characterize all the unknown impurities that are present at a level of >0.1% [9–10]. Though synthesis and separation of triclabendazole intermediates were largely overlooked [11–12], the impurities profile study of triclabendazole was not reported to the best of our knowledge. The purpose of this study, therefore, was to develop a simple method for the isolation and characterization of impurities present in the triclabendazole bulk drug which is of great importance not only for quality control but also monitoring of reactions during process development of benzoimidazole anthelmintic agents.

2. Experimental

2.1. Samples and chemicals

The investigated samples including bulk drug triclabendazole and its intermediates produced in multi-step synthetic reactions were obtained from Jiouchou Pharm.

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doi:10.1016/j.jpba.2004.09.049

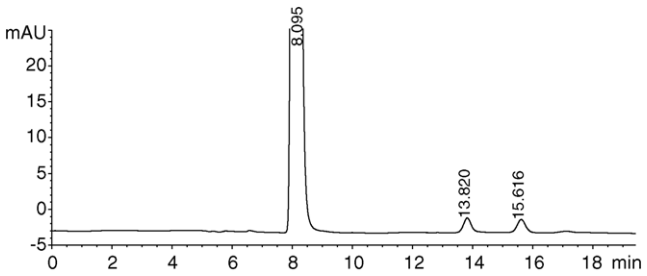


Fig. 1. HPLC chromatogram of the triclabendazole bulk drug. For the chromatographic conditions, see Section 2.2.

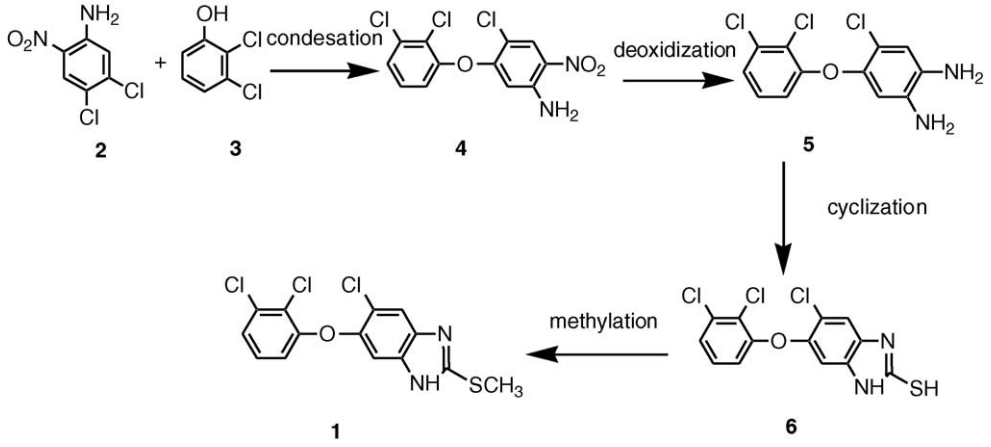
Inc., Hangzhou, China. Acetonitrile and methanol used for analytical and preparative HPLC were of chromatographic grade and purchased from Merck, Darmstadt, Germany. Deionized water (18 MΩ) was obtained from a Milli-Q purification system, Millipore, Bedford, MA, USA.

2.2. High-performance liquid chromatography (analytical)

The analytical HPLC was performed using an Agilent 1100 chromatography system including a G1312 BinPump, G1314A variable-wavelength detector (VWD), model 7725 injector fitted with 20 μl sample loop, a PT100 column oven and an Agilent ChemStation for LC. The column used was a reversed-phase C18 column (YMC-PACK ODS-A, 150 mm × 4.6 mm i.d., 5 μm, 120 Å) at 25 °C. The mobile solvent was acetonitrile and water (60:40, v/v). The flow-rate was 1.0 ml/min, and the effluent was monitored at 230 nm.

2.3. High-performance liquid chromatography (semi-preparative)

A Waters Delta 600 semi-preparative chromatography system equipped with Waters 600 controller, Waters 2996 photodiode array detector (PAD), Rheodyne Injector Model 7725i with 5.0 ml loop and Millennium^{32} Chromatography Manager for LC were used. The semi-preparative separation was performed with a reversed-phase column (Symmetry® C18, 100 mm × 7.8 mm i.d., 5 μm, 100 Å) using methanol and water (75:25, v/v) as mobile solvent at a flow-rate of 4 ml/min at 25 °C, and an UV detection at 230 nm.



Scheme 1. Scheme for the synthesis of triclabendazole.

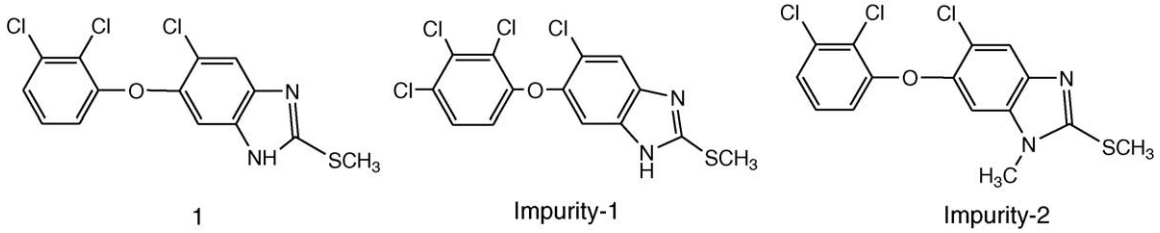


Fig. 2. Structures of triclabendazole and its related impurities.

2.4. NMR spectroscopy

1D and 2D NMR experiments were performed at 298 K using DMSO-d_6 as solvent and TMS as internal standard on a Bruker Advance DMX 500 instrument with a 5 mm QNP probe head, and analyzed on Silicon Graphics O2 workstations using XWINNMR version 2.1 (Bruker Analytik GmbH, Germany). ^{1}H NMR spectra were recorded at a proton frequency of 500.13 MHz with a spectral width of 15.02 kHz. The acquisition time was 1.1 s and relaxation delay 1 s; 64 scans with 32K data points were used. The ^{13}C NMR spectra were obtained using a spectral width of 31 kHz; 8192 scans with 32 K data points were used. The chemical shifts were referenced to DMSO. The one-bond

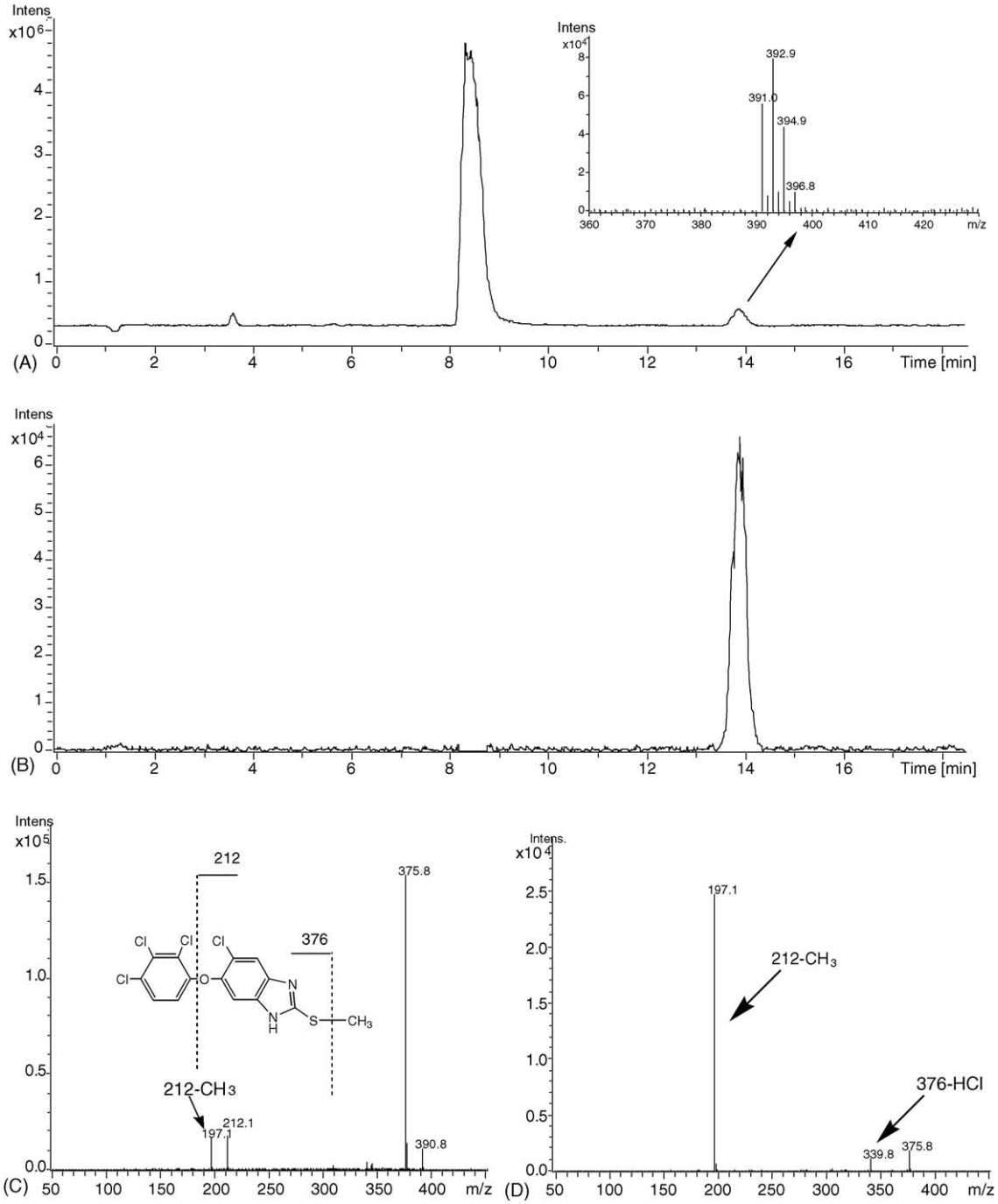


Fig. 3. Negative ion mode HPLC/ESI-MS analyses of impurity-1. (A) TIC and ESI-MS; (B) EIC of *m/z* 391; (C) MS^2 on precursor ion *m/z* 391; (D) MS^3 on product ion *m/z* 376.

Table 1
1D and 2D NMR data of impurity-1

Atom	^{13}C NMR δ (ppm)	DEPT[a]	HMQC δ (ppm)	HMBC correlation with	^{1}H–^{1}H COSY correlation with
2	164.8	q[b]		SCH_3	
3a	148.0	q		H-7	
4	113.7	CH	7.28 (1H, s)		
5	115.0	q		H-7	
6	155.1	q			
7	106.8	CH	7.05 (1H, s)		
7a	140.0	q		H-4	
SCH_3	14.0	CH_3	2.66 (3H, s)		
1′	156.0	q		H-5′	
2′	122.3	q		H-6′	
3′	132.0	q		H-5′	
4′	125.5	q		H-6′	
5′	128.8	CH	7.50 (1H, d, J = 9.12 Hz)		H-6′
6′	114.4	CH	6.51 (1H, d, J = 9.16 Hz)		H-5′

[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

heteronuclear correlation (HMQC) spectra were obtained using the invieags program in the Bruker software. The spectra resulted from a 256 × 1024 data matrix with eight scans per t_1 increment. The acquisition time was 0.085 s. The long-range ^{1}H–^{13}C correlation (HMBC) spectra were obtained using the inv4gslrnd program in the Bruker software. The spectra resulted from a 256 × 1024 data matrix with 16 scans per t_1 increment. The acquisition time was 0.085 s.

2.5. Mass spectrometry (ESI-MS^n, GC/MS)

ESI-MS^n analysis has been performed on Bruker Esquire 3000^{plus} mass spectrometer equipped with an ESI interface, ion trap analyzer system and acquired data in software Esquire 5.0. The ion source temperature was 250 °C, and the source voltage was always set at 3.5 kV. Nitrogen was used as the sheath and nebulization gas set at 6 l min^{-1} and 20 psi, respectively. Helium was introduced into the system to an estimated pressure of 6×10^{-6} mbar to improve trapping efficiency, and also provided as the collision gas during the MS^n experiment. For MS/MS spectra, the mass spectrometer was tuned optimizing the specific collision energy between 0.5 and 1.1 V to maximize the ion current of sequential MS^n of triclabendazole and two impurities. The mass spectra were acquired with a scan rate of 13,000 u s^{-1}.

Gas chromatography/mass selective detector (GC/MSD) analyses were performed on a ThermoQuest Trace 2000 GC-MSD using a 30 m × 0.32 mm i.d. HP-5MS fused-silica capillary column (95% dimethyl–5% diphenyl polysiloxane) with a 0.25 μm coating thickness with helium as the carrier gas at a constant flow rate of 1 ml min^{-1}. The operating conditions for the analyses were: manual injection, split 1:10; injection temperature, 280 °C; initial temperature 60 °C for 3 min, programming 10 °C min^{-1} to a final temperature of 250 °C for a total run time of 26 min. During full-scan acquisition the mass spectra were collected over a mass range from 30 to 500 amu using 70 eV electron energy; detector voltage 350 V; interface temperature 260 °C. MSD were tuned before each injection using PFTBA (perfluorotributylamine) as tuning standard. The used MS library was NIST2.0 with 140,000 database.

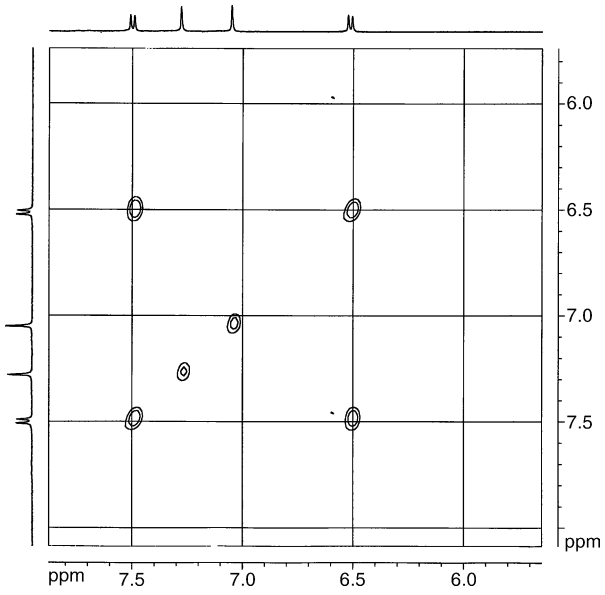


Fig. 4. ^{1}H–^{1}H COSY spectrum of impurity-1.

2.6. Synthesis of triclabendazole

Scheme 1 shows the sequence leading to triclabendazole [11,12]. Cross coupling reaction of 2-nitro-4,5-dichloroaniline (**2**) and 2,3-dichlorophenol (**3**) afforded the key intermediate 4-chloro-5-(2,3-dichlorophenoxy)-2-nitroaniline (**4**), which was reduced with Fe to give phenyldiamine (**5**). Reaction with carbon disulfide and methyliodine gives the desired product triclabendazole.

3. Results and discussion

3.1. Detection of impurities 1 and 2

The triclabendazole bulk drug was first analyzed by analytical HPLC. As shown in Fig. 1, the contents of impurity-1 at retention time of 13.82 min and impurity-2 at retention time of 15.62 min were up to 0.1% based HPLC peak area percentage. The structures of these impurities and triclabendazole are shown in Fig. 2.

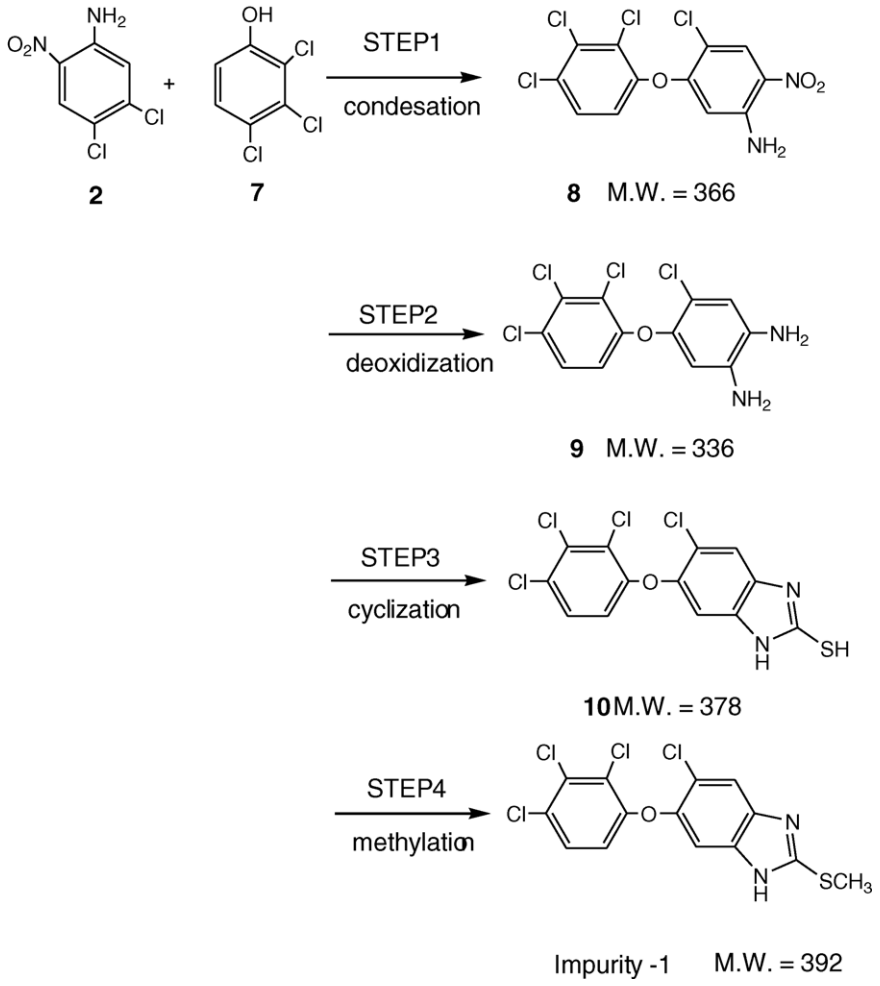
3.2. Isolation of impurities by semi-preparative HPLC

For the isolation of the impurity-1 and impurity-2, the semi-preparative HPLC has been performed under the condition described in Section 2.3. All the fractions of impurities isolated were evaporated to dryness and used to generate spectra data.

3.3. Structural elucidation

3.3.1. Structure elucidation of impurity-1 and its intermediates

On-line high-performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS) chromatography in negative ion mode (Fig. 3A and B) displayed that the impurity-1 at the retention time of



Scheme 2. Scheme for the synthesis of the intermediates of impurity-1.

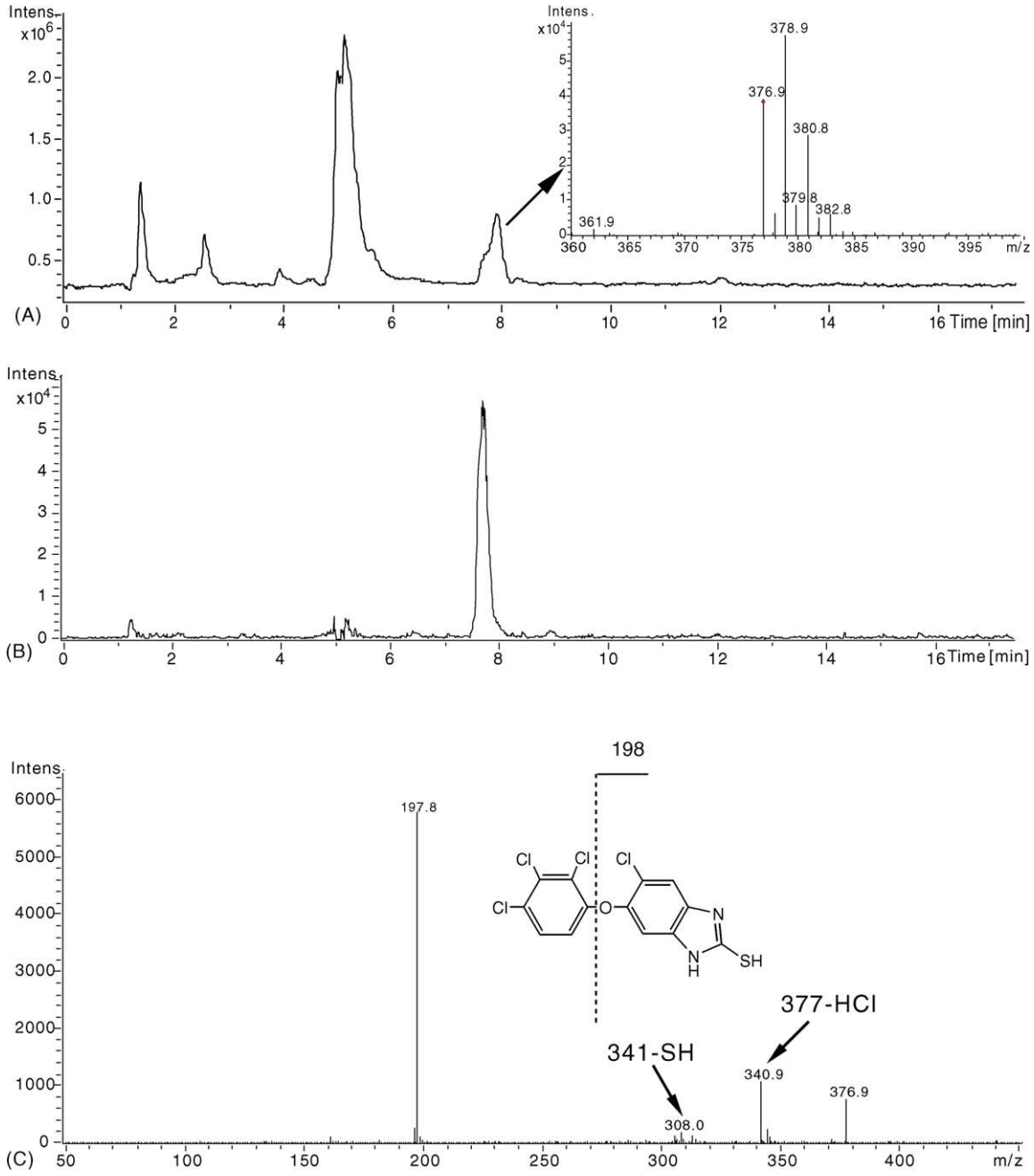


Fig. 5. Negative ion mode HPLC/ESI-MS analyses of intermediate **10**. (A) TIC and ESI-MS; (B) EIC of *m/z* 377; (C) MS^2 on precursor ion *m/z* 377.

13.820 min has five deprotonated isotope distribution ions at *m/z* 391, 393, 395, 397 and 399 which are close in agreement with the calculated ratio of 81:108:54:12:1 indicating that the impurity-1 has four chlorine atoms. After scanning the fragment of *m/z* 391 with collision-induced dissociation (CID), the product ion spectrum showed the characteristic fragment ions at *m/z* 376, 212, and 197 (Fig. 3C). The MS^3 at *m/z* 376 showed two characteristic fragment ions at *m/z* 340 and 197 (Fig. 3D). The fragment ion at *m/z* 197 was attributed to 6-chloro-2-mercapto-3*H*-benzoimidazol-5-ol. The product ion at *m/z* 340 is due to lose of HCl from the precursor ion at *m/z* 376 [13]. These MS^n fragment ions were close in agreement with the structure of impurity-1.

1D and 2D NMR data (Table 1) revealed that the impurity-1 has similar structure with trclabendazole. ^1H

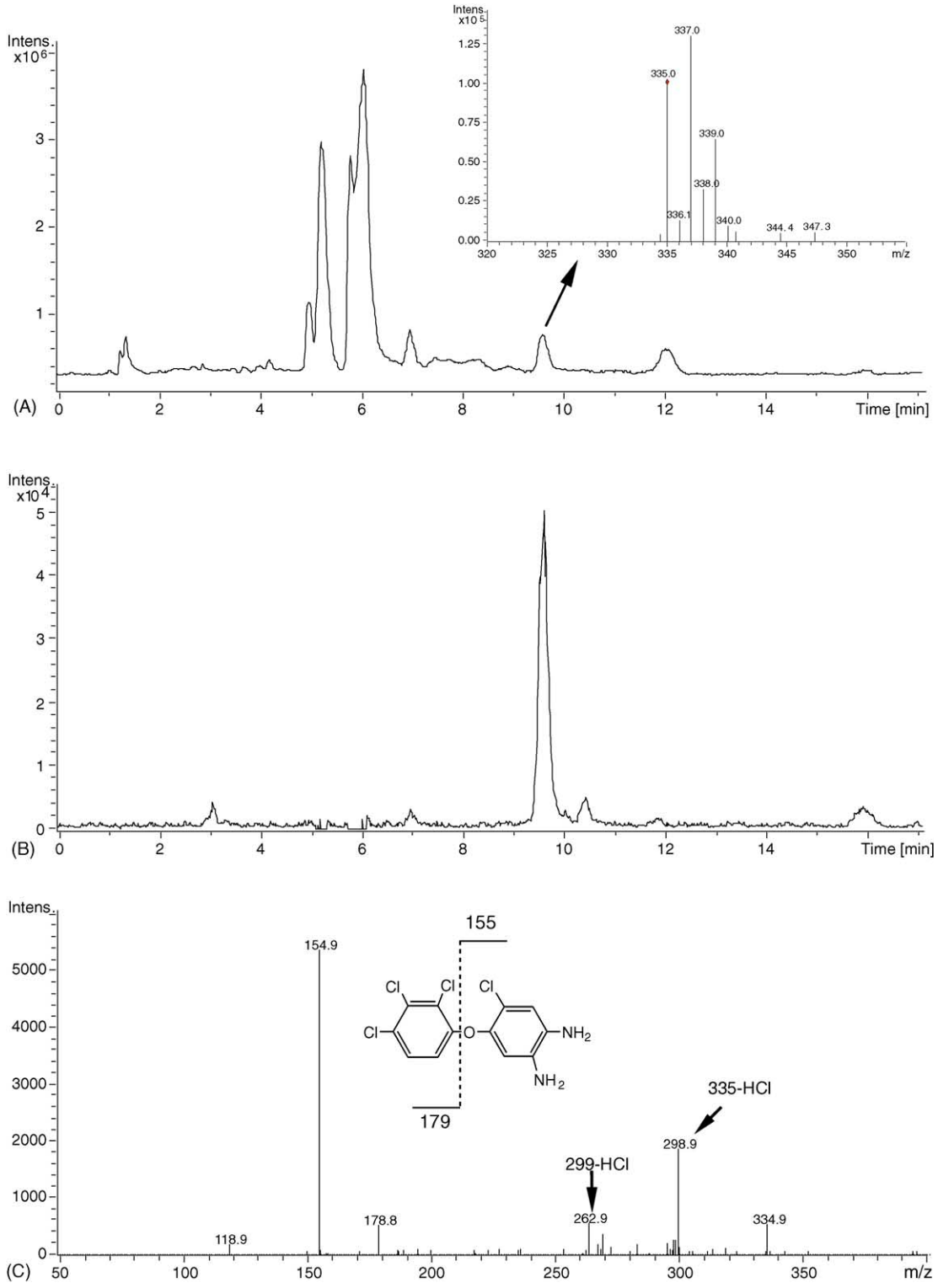


Fig. 6. Negative ion mode HPLC/ESI-MS analyses of intermediate **9**. (A) TIC and ESI-MS; (B) EIC of *m*/*z* 335; (C) MS^2 on precursor ion *m*/*z* 335.

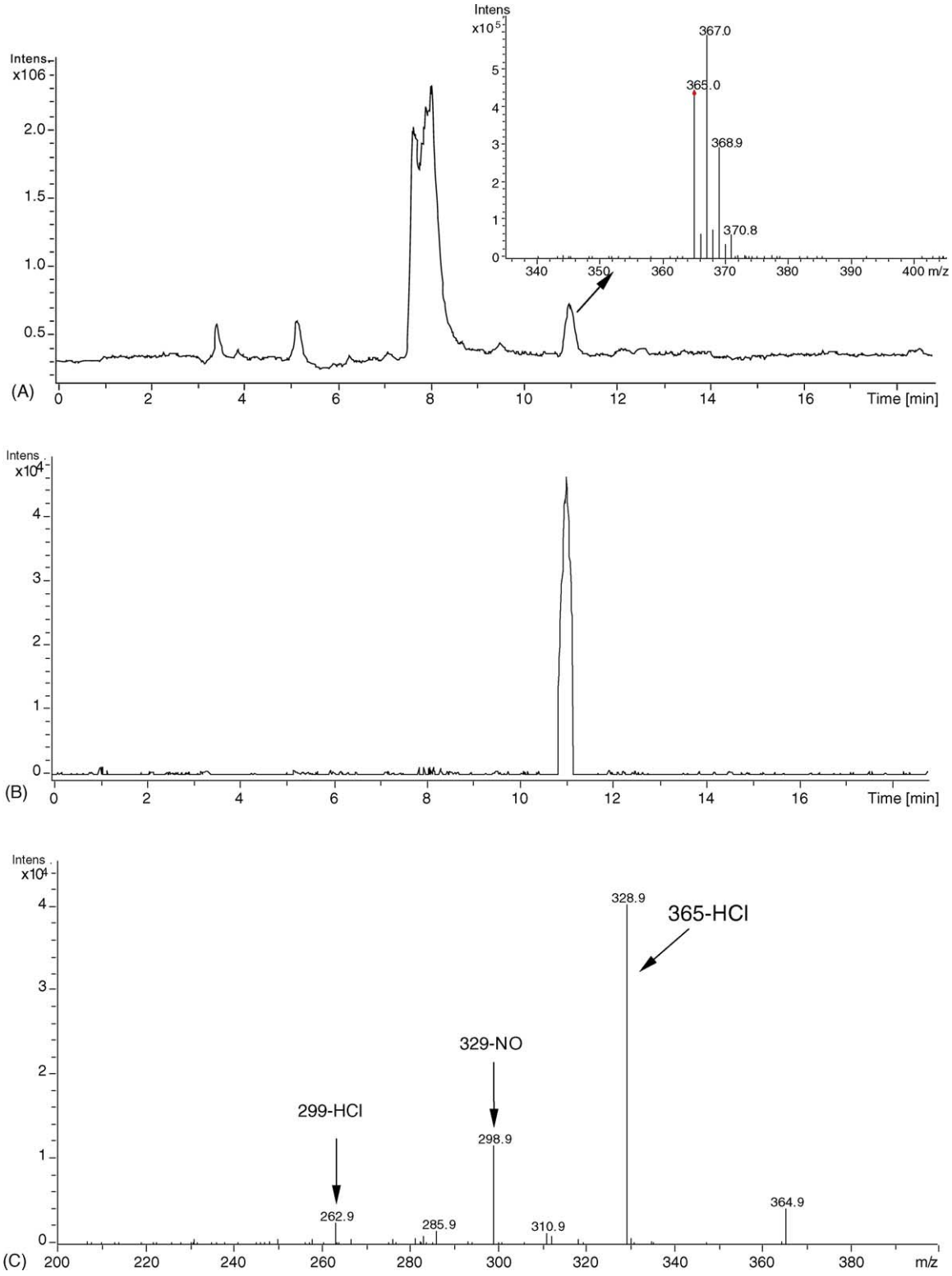


Fig. 7. Negative ion mode HPLC/ESI-MS analyses of intermediate **8**. (A) TIC and ESI-MS; (B) EIC of *m*/*z* 365; (C) MS^2 on precursor ion *m*/*z* 365.

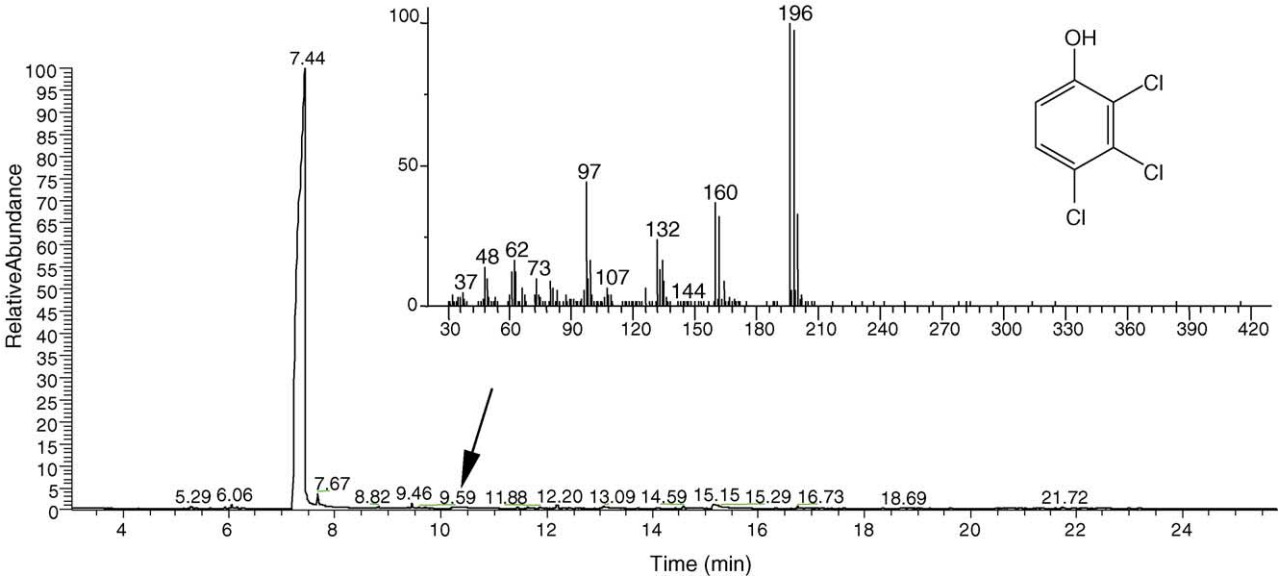


Fig. 8. GC/EI-MS analyses of starting impurity **7**.

NMR spectrum indicated the impurity-1 has four aromatic protons: two single proton signals at δ 7.28 and 7.05 and two duple signals at δ 7.50 and 6.51. ^{1}H–^{1}H COSY spectrum (Fig. 4) clearly showed the cross-peaks between the two duple proton signals at δ 7.50 and 6.51 ppm indicating only two protons are closely related, which hints that there are two possible structures, such as 5-chloro-6-(2,3,4-trichloro-phenoxy)-2-methylsulfanyl-1*H*-benzoimidazole and 5-chloro-6-(2,3,6-trichloro-phenoxy)-2-methylsulfanyl-1*H*-benzoimidazole. However, HMQC spectrum of impurity-1 shows that the proton at δ 7.50 ppm is directly correlated to the resonance at δ 128.8 ppm and the proton at 6.51 ppm is directly correlated to the resonance at 114.4 ppm, which indicate that impurity-1 has a 2,3,4-trichloro-phenoxy derivate not a 2,3,6-trichloro-phenoxy substitution [14]. HMBC data (Table 1) also provide enough information to support the structure. Therefore, all data including ESI-MS^n, 1D and 2D NMR data suggest that the impurity-1 is 5-chloro-6-(2,3,4-trichlorophenoxy)-2-methylsulfanyl-1*H*-benzoimidazole.

According to the reaction sequence of triclabendazole (Scheme 1), it is presumable that impurity-1 possibly produced in same reaction mechanism (Scheme 2). Then we used on-line HPLC/ESI-MS with the EIC techniques to track the corresponding by-products in every synthesis steps. Combined with the TIC and EIC technique in HPLC/ESI-MS, we detected the compound **10** (*m*/*z*, 377, 379, 381, 383, 385) (Fig. 5A and B), compound **9** (*m*/*z*, 335, 337, 339, 341, 343) (Fig. 6A and B), and compound 8 (*m*/*z*, 365, 367, 369, 371, 373) (Fig. 7A and B). The MS^2 spectra of compounds **8** (Fig. 7C), **9** (Fig. 6C) and **10** (Fig. 5C) are also close in agreements with their structures.

By means of GC/MS analyses, the impurity in crude material of step 1 was detected and confirmed. GC analysis showed the crude material 2,3-dichlorophenol (**3**) contained 0.46% content of impurity **7**. GC/MS (Fig. 8) and data matching confirmed the structure of **7** as 2,3,4-trichlorophenol.

Thus, formation of impurity-1 was rationalized by the presence of 2,3,4-trichlorophenol (**7**) that a similarity of 2,3-dichlorophenol (**3**), which is the starting compound in the synthesis of triclabendazole. It is condensed with 2-nitro-4,5-dichloroaniline **(2)** to give **8**, then reduced to **9,** following cyclized to give **10**, and yielded the impurity-1 by methylation in the last step.

3.3.2. Structure elucidation of impurity-2

HPLC/ESI-MS chromatogram and spectra using the positive ion mode were showed the impurity-2 at the retention time of 15.616 min has a characteristic protonated isotope pattern at *m*/*z* 373, 375, 377, 379 (Fig. 9), indicating the presence of three chlorine atoms in the molecule. The measured molecular weight is 14 mass units greater than triclabendazole, this can be attributed to the methyl incorporation in triclabendazole. The methyl substitution on group NH was further confirmed from the singlet signal at δ 3.34 (3H, CH_3) and the absence of singlet at δ 12.86 (1H, NH) in ^{1}H NMR spectrum. It is produced in the last synthesis procedure of Scheme 1.

These data confirmed the molecular formular of impurity-2 as $C_{15}H_{11}Cl_3N_2OS$ corresponding its structure as 5-chloro-6-(2,3-dichlorophenoxy)-1-methyl-2-methylsulfanyl-1*H*-benzoimidazole (Fig. 2).

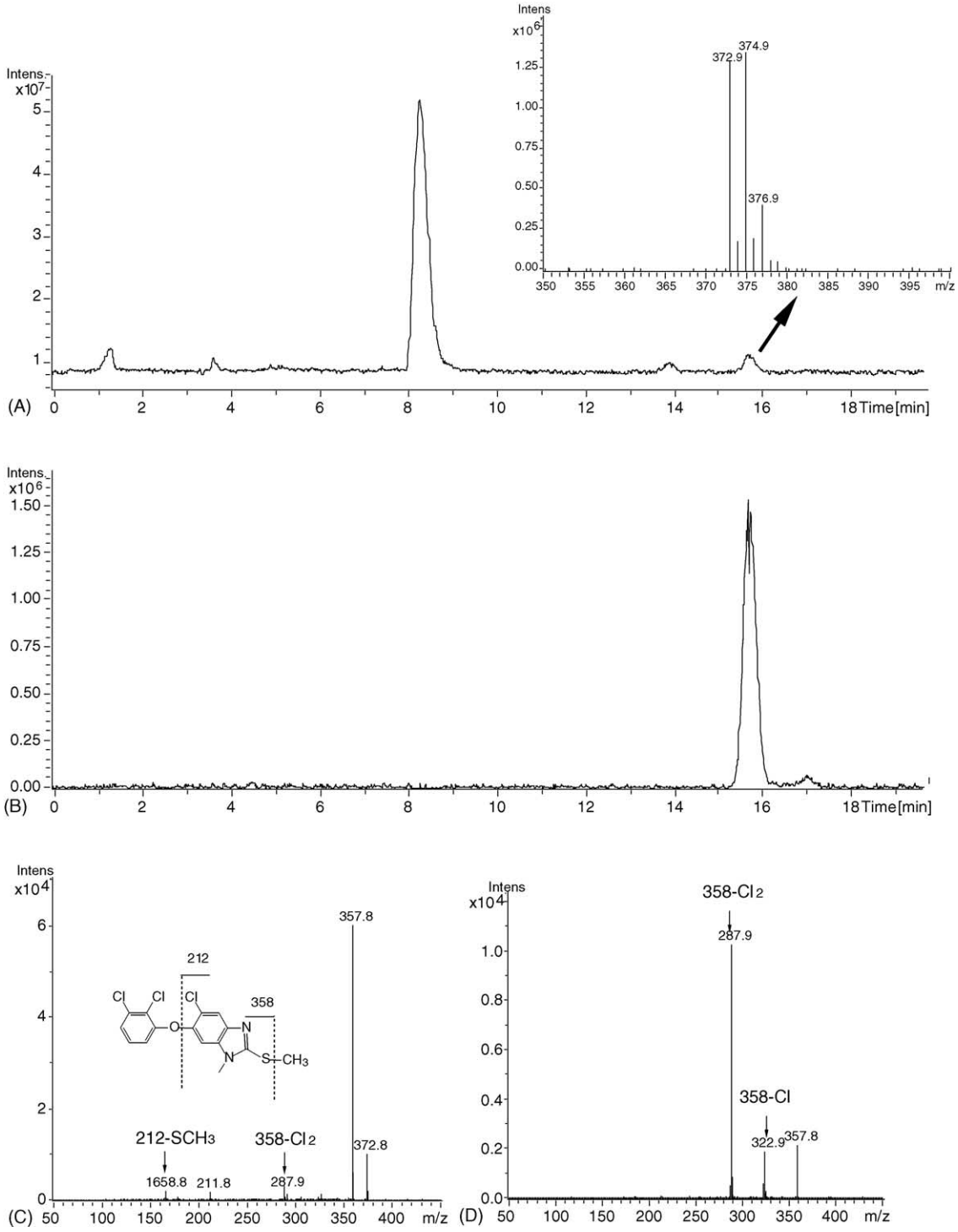


Fig. 9. Positive ion mode HPLC/ESI-MS analyses of impurity-2. (A) TIC and ESI-MS; (B) EIC of *m*/*z* 373; (C) MS^2 on precursor ion *m*/*z* 373; (D) MS^3 on product ion *m*/*z* 358.

4. Conclusions

A simple and efficient on-line reversed-phase HPLC/ESI-MS method has been developed for the separation and characterization of synthetic impurities of triclabendazole. TIC, EIC and MS^n were successively applied to study the structures of the traced impurities. The method also detected and confirmed some intermediates of impurity-1.

Acknowledgements

Research was supported in part by the Natural Science Foundation of China (20375036) and Zhejaing Province (RC0042).

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