



Separation and characterization of allergic polymerized impurities in cephalosporins by 2D-HPSEC $\times$ LC-IT-TOF MS



Yu Xu^a, DanDan Wang^a, Lan Tang^a, Jian Wang^{a,b,*}

^a Zhejiang University of Technology, Hangzhou 310014, China

^b Zhejiang Institute for Food and Drug Control, Hangzhou 310052, China

ARTICLE INFO

Article history:

Received 5 June 2017

Received in revised form 30 July 2017

Accepted 31 July 2017

Available online 2 August 2017

Keywords:

Polymerized impurity

Cephalosporin

High performance size exclusion chromatography

2D-HPSEC $\times$ LC-IT-TOF MS

ABSTRACT

Eleven unknown allergic impurities in cefodizime, cefmenoxime and cefonicid were separated and characterized by a trap-free two-dimensional high performance size exclusion chromatography (HPSEC) and reversed phase liquid chromatography (RP-HPLC) coupled to high resolution ion trap/time-of-flight mass spectrometry (2D-HPSEC $\times$ LC-IT-TOF MS) with positive and negative modes of electrospray ionization method. Separation and characterization the allergic polymerized impurities in β -lactam antibiotics were on the basis of column-switching technique which effectively combined the advantages of HPSEC and the ability of RP-HPLC to identify the special impurities. In the first dimension HPSEC, the column was Xtimate SEC-120 analytical column (7.8 mm $\times$ 30 cm, 5 μ m), and the gradient elution used pH 7.0 buffer-acetonitrile as mobile phase And the second dimension analytical column was ZORBAX SB-C18 (4.6 $\times$ 150 mm, 3.5 μ m) with ammonium formate solution (10 mM) and ammonium formate (8 mM) in [acetonitrile-water (4:1, v/v)] solution as mobile phase. Structures of eleven unknown impurities were deduced based on the high resolution MSⁿ data with both positive and negative modes, in which nine impurities were polymerized impurities. The forming mechanism of β -lactam antibiotic polymerization in cephalosporins was also studied. The question on incompatibility between non-volatile salt mobile phase and mass spectrometry was solved completely by multidimensional heart-cutting approaches and online demineralization technique, which was worthy of widespread use and application for the advantages of stability and repeatability.

© 2017 Published by Elsevier B.V.

1. Introduction

Cephalosporin, broad-spectrum β -lactam antibiotics, are widely used in human and animal therapy [1]. Polymerized impurities of cephalosporins are generated easily during production and storage of the material [2]. A large number of clinical trials and studies have confirmed that polymerized impurities in the β -lactam antibiotics can cause immediate hypersensitivity, so determination and control of polymerized impurities in the β -lactam antibiotics is extremely important [3].

Since the polymerized impurity of β -lactam antibiotics is very unstable, it is very difficult to prepare and standardize the impurity reference substance. The British Pharmacopoeia (BP 2009) uses RP-HPLC for identification of the polymerized impurities peaks of

amoxicillin, ampicillin, cefotaxime sodium by comparison of their relative retention times (RT) between drugs and polymers. However, the practice proved that using RP-HPLC for identification of the polymerized impurities is problematic due to the difficulty to obtain polymerized impurities reference substance [4]. Polymerized impurities of cephalosporins including cefotaxime, ceftriaxone, cefoperazone and ceftazidime are controlled by a gel filtration chromatography (GFC) in Chinese Pharmacopoeia (ChP) [5]. At present, the most common method for determination the polymer in cephalosporin is Sephadex G-10 gel chromatography, which shows low column efficiency, poor separation and often need a longer time for analysis [6]. Compared with Sephadex G-10 gel chromatography, high performance gel chromatography has the advantages of high sensitivity, good separation and short analysis time. In recent years, high performance gel chromatography has been widely used in the detection of the polymerized impurities in cephalosporin because of its better separation ability, short analysis time and high sensitivity [7,8].

* Corresponding author at: Zhejiang Institute for Food and Drug Control, Hangzhou 310052, China.

E-mail address: wangjianhw2000@aliyun.com (J. Wang).

Based on the request of ICH, the structures of impurities whose content are over 0.1% need to be confirmed. Therefore, it is necessary to characterize the structures of polymerized impurities in cephalosporin that has a great significance in improving the quality of cephalosporin [9]. Liquid chromatography-mass spectrometry (LC–MS) has evolved as an identification tool for the characterization of drug impurities and degradation products [10]. IT-TOF-MS combines the multistage fragmentation function of ion trap full scan mode with high resolution and sensitivity and the accurate determination of molecular weight by TOF mass spectrometry, which greatly improves the ability of accurate tracing components in a complex matrix.

However, all the above LC–MS methods were based on volatile mobile phase. In non-volatile systems, the selectivity and sensitivity were limited. A non-volatile system is used in the official analytical method of Chinese Pharmacopoeia for detection of the polymerized impurities in cephalosporin. Thus, the characterization of unknown peaks in a non-volatile system, based on data obtained from a volatile LC–MS method, is problematic [11]. Two-dimensional liquid chromatography-mass spectrometry, which has undergone a dramatic development over the last decade, can solve these problems. In the multidimensional heart-cutting approaches (LC–LC), the first chromatographic dimension (1D) can be applied to trapped the aimed impurities by valve-switching and stored in 20 μ l quantitative loop using the mobile phase with non-volatile salt, then the second chromatographic dimension (2D) removes the non-volatile salt using methanol and pure water as mobile phase [12,13], leading to TIC chromatogram of LC–MS consist with the LC chromatogram of the official analytical method in the peak sequence of impurities. Jian Wang et al. characterized the oxidation degradation products in tigecycline by a two-dimensional liquid chromatography combined with Q orbitrap mass spectrometry [11]. Because the mobile phase of non-volatile buffer solution did not need to be replaced by volatile buffer solution, the pharmacopeia methods using non-volatile buffer were applicable to LC–MS methods, which lead either the shorter researching time or the risk of missing some impurities due to the retention time variation was avoided. However, the efficiency of this method would be reduced due to the technique only had one loop. Jinlin Zhang et al. used trap-free two-dimensional liquid chromatography to identify the impurities in doxycycline hyclate [14]. Since trap-free two-dimensional liquid chromatography which means do not need a trap column and has six quantitative loops that can handle five impurities simultaneously, making the analysis method more rapid, convenient and time-saving than other analytical methods of two-dimensional liquid chromatography.

In this study, structures of eleven impurities in cefodizime, cefmenoxime and cefonicid were characterized by trap-free 2D-HPSEC $\times$ LC-IT-TOF MS with positive and negative electrospray ionisation modes. Each peak eluted from the non-volatile system (one-dimension gel analytical column) was trapped by valve-switching and stored in a 20 μ l quantitative loop, then sent to the volatile mobile phase (two-dimension C18 analytical column), which is connected to MS. Structures and fragment pathways of eleven unknown impurities were investigated by complete fragmentation patterns, in which nine impurities were polymerized impurities. And the forming mechanism of β -lactam antibiotic polymerization in cephalosporins was also studied. This method had provided the basis of the separation and analysis of β -lactams polymerized impurities, and it could be used to improve the quality of product. To date, there is no report concerning characterization and forming mechanisms of polymerized impurities of cefodizime, cefmenoxime and cefonicid. Hence present research work is undertaken considering general interest.

2. Experimental

2.1. Materials

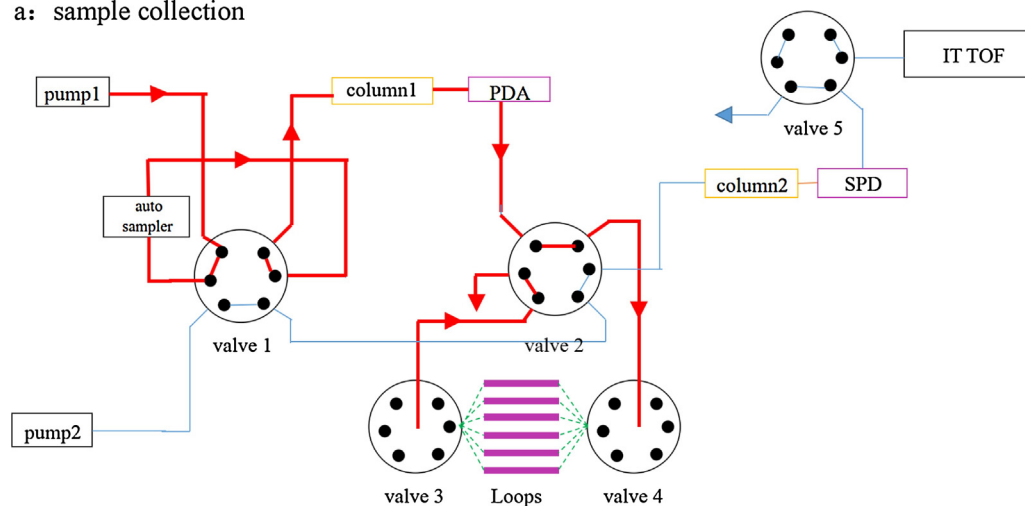
Cefodizime (batch number: 1609241) used in this study was obtained by Zhejiang Jingxin Pharmaceutical Co. Ltd. (Shaoxing, China). Cefonicid (batch number: 060401) used in this study was obtained by Zhejiang Huidisen Pharmaceutical Co. Ltd. (Hangzhou, China). Cefmenoxime (batch number: 20101018) used in this study was obtained by Zhejiang Huidisen Pharmaceutical Co. Ltd. (Hangzhou, China). Acetonitrile was purchased from Merck (Darmstadt, Germany), ammonium formate was purchased from Sigma-Aldrich (St. Louis, MO, USA), dibasic sodium phosphate (Analytical reagent), sodium dihydrogen phosphate (Analytical reagent) and Sodium carbonate (Analytical reagent) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), and water was HPLC grade (generated by a Millipore Milli-Q-Gradient purification system).

2.2. Instrumentation

2.2.1. Trap-free two-dimension LC apparatus

A trap-free two-dimension Nexera-XR liquid chromatograph (Shimadzu, Kyoto, Japan), a system equipped with a binary pump $\times 2$ was connected to a Shimadzu SIL-30AC autosampler. The first dimension included a binary pump (LC-30AD), auto-sampler (SIL-30AC), column thermostat (CTO-20A) and diode array detector (SPD-M20A). Chromatographic separation in the first dimension chromatography was carried out at 40 °C using a Xtimate SEC-120 analytical column (7.8 mm $\times$ 30 cm, 5 μ m). The mobile phase of 1D-LC: pH 7.0 buffer [0.005 mol/L dibasic sodium phosphate solution – 0.005 mol/L sodium dihydrogen phosphate solution (61:39, v/v)]-acetonitrile with gradient conditions: 0 min 5% B (hold for 15 min); 18 min, 20% B (hold for 2 min); 23 min, 5% B (hold for 3 min). The mobile phase flow rate was 0.80 mL min⁻¹ and injection volume was 30 μ L. The second dimension was consisted of a binary pump (LC-20AD), column thermostat (CTO-20A) and UV/VIS detector (SPD-20A). The mobile phase of 2D chromatography of method A (the chromatogram was shown in Fig. S1(A)): (A) ammonium formate solution (10 mM) and (B) acetonitrile with gradient conditions: 0 min 5% B; 5 min, 95% B; 5.5 min, 5% B; 9 min, 5% B, using a Shimadzu Shim-pack GISS C18 analytical column (50 mm $\times$ 2.1 mm, 1.9 μ m). The mobile phase of 2D chromatography of method B (the chromatogram was shown in Fig. S1(B)): (A) acetic acid solution (0.1%, v/v) and (B) acetonitrile with gradient conditions: 0 min 5% B (hold for 10 min); 12 min, 15% B (hold for 13 min); 45 min, 60% B (hold for 10 min); 55.1 min, 5% B (hold for 10 min), using a ZORBAX SB-C18 analytical column (4.6 $\times$ 150 mm, 3.5 μ m). The mobile phase of 2D chromatography of method C (the chromatogram was shown in Fig. S1(C)): (A) ammonium formate solution (10 mM) and (B) ammonium formate (8 mM) in [acetonitrile-water (4:1, v/v)] solution with gradient conditions: 0 min 12% B; 9.3 min, 16% B; 15 min, 20% B; 18 min, 40% B; 19 min, 12% B (hold for 10 min), using a ZORBAX SB-C18 analytical column (4.6 $\times$ 150 mm, 3.5 μ m). The mobile phase flow rate was 0.40 mL min⁻¹. The column temperature was at 40 °C and the detection wavelength was 254 nm in the first and second dimension. The first and second dimension columns were connected by means of two high speed/high pressure six-position and six-port switching valves, and equipped with six 20 μ L stainless steel loops. Fig. 1 showed the schematic plot of instrument structure. In Fig. 1a, the switch valve 1 and valve 2 were set to the “sample collection”, and sample were directly transferred to the 20 μ L quantitative loops with non-volatile mobile phase by control of valve 3 and valve 4. The system retained this configuration for 26 min. After, the switch valve 1 and valve 2 was turned to the “LCMS analysis” (Fig. 1b), thus

a: sample collection



b: LCMS analysis

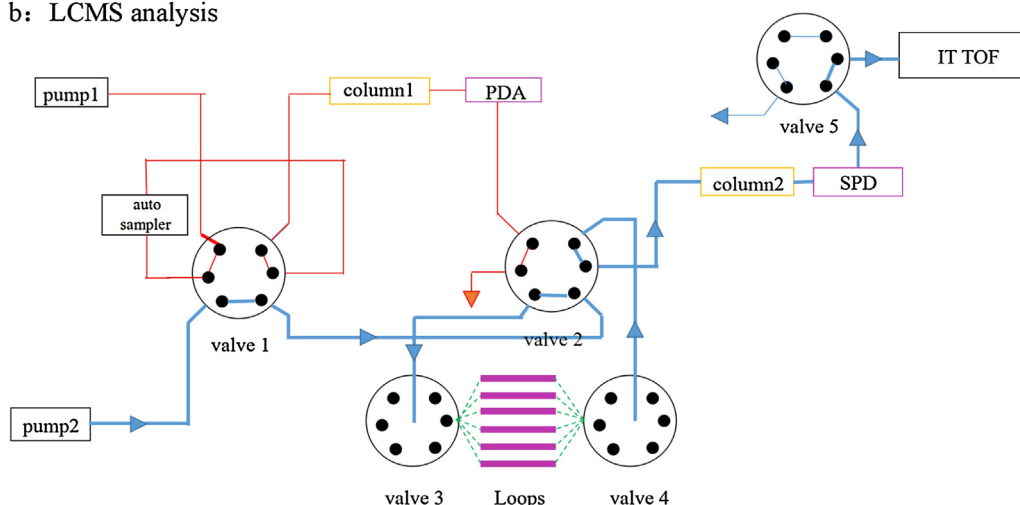


Fig. 1. A schematic of the instrumental set-up used for LC × LC–IT–TOF MS analyses, using two six-position, six-port valves equipped with six 20 μL sample loops.

connecting ion trap/time-of-flight mass spectrometer. The trapped analytes in quantitative loops were transferred to the second analytical column with the volatile mobile phase. Whether substances drain to MS or drain to waste was controlled by the valve 5.

2.2.2. MS spectrometry

Trap-free two-dimensional liquid chromatography was coupled to ion trap/time-of-flight mass spectrometer (2D LC–IT–TOF MS) from Shimadzu Corp. (Kyoto, Japan). The mass spectrometry detector (MSD) was equipped with an electrospray ionization (ESI) source. The ionization mode was positive and negative alternatively. The interface and MS parameters were as the follows: nebulizer gas flow: 1.5 L min^{-1} (nitrogen); Collision gas: argon; the temperature of curve desolution line (CDL) and heat block: 200°C ; detector voltage: 1.56 V ; scan range: $100\text{--}2000 \text{ m/z}$; the mode of MS^n : manual to MS^n .

The structures of cefodizime, cefonicid and cefmenoxime presented both amino and carboxylic groups, leading to the response in the mass spectrometry either in the positive ionization or in the negative ionization, so in this study we used the both modes to predict and verify the structures of cefodizime, cefonicid, cefmenoxime and their impurities.

2.2.3. Software

All data acquired were processed by Shimadzu LC-solution software (Kyoto, Japan). Formula predictor from Shimadzu Corp. (Kyoto, Japan) was used to simulate and study the fragmentation behavior of the described compounds.

2.2.4. Sample preparation

10 mg of efodizime sodium was dissolved with water to make a solution of 10 mg mL^{-1} . 10 mg of cefonicid sodium was dissolved with water to make a solution of 10 mg mL^{-1} . And 10 mg of cefmenoxime hydrochloride was dissolved with 5 mg mL^{-1} sodium carbonate solution to make a solution of 10 mg mL^{-1} .

3. Results and discussion

3.1. Selection of chromatographic conditions

The non-volatile mobile phase in the official analytical method of Chinese Pharmacopoeia for cefodizime was used as the mobile phase in the first dimension system. Cefodizime, cefonicid and cefmenoxime could be completely separated from various degradation products and impurities. To permit the use of liquid chromatography-mass spectrometry analysis, the mobile phase in the second dimension system must be a volatile, and concentration of salt should be as low as possible. Fig. S1 showed the second

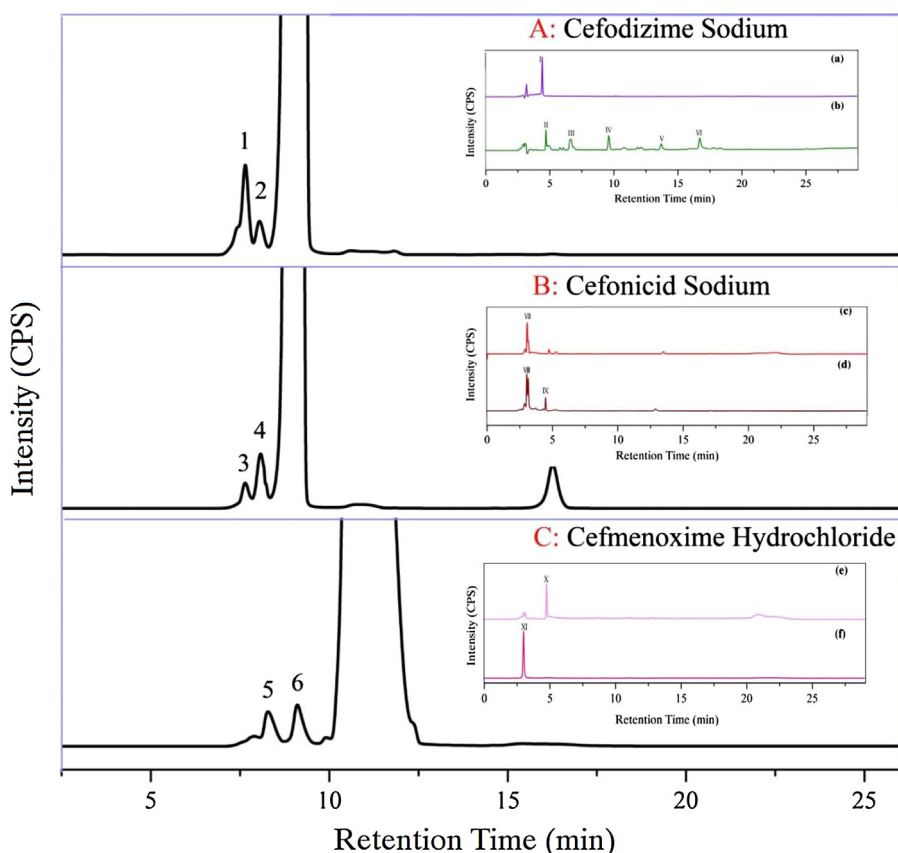


Fig. 2. First dimension gel chromatography of cefodizime sodium(A), cefonicid sodium(B) and cefmenoxime hydrochloride(C). The insert pictures were the second dimension RPLC-UV of six polymer peaks, a- Peak 1; b- Peak 2; c- Peak 3; d- Peak 1; e- Peak 2; f- Peak 3. λ of detection = 254 nm.

Table 1

ESI-MS exact mass data and theoretical mass data on $[M+H]^+$ and $[M-H]^-$ of eleven impurities of cefodizime, cefmenoxime and cefonicid in the positive and negative ion modes.^a

Imp.	formula	$[M+H]^+$ (m/z)	$[M-H]^-$ (m/z)	Theoretical $[M+H](m/z)$	Theoretical $[M-H](m/z)$	Deviation (ppm)
I	C ₃₄ H ₃₅ N ₁₁ O ₁₃ S ₆	998.0796	996.0640	998.0813	996.0667	0.80/-2.71
II	C ₂₈ H ₂₈ N ₈ O ₁₀ S ₅	797.0620	795.0454	797.0605	795.0459	1.88/-0.62
III	C ₂₈ H ₂₇ N ₈ O ₁₀ S ₅	731.0367	729.0246	731.0387	729.0241	-2.74/0.89
IV	C ₃₄ H ₃₃ N ₁₁ O ₁₂ S ₆	980.0700	978.0586	980.0707	978.0562	-0.71/2.45
V	C ₃₄ H ₃₃ N ₉ O ₁₁ S ₇	968.0405	966.0271	968.0417	966.0272	-1.24/-0.10
VI	C ₃₆ H ₃₉ N ₁₁ O ₁₃ S ₆	1026.1127	1024.0967	1026.1126	1024.0980	-0.10/-1.56
VII	C ₁₈ H ₂₆ N ₈ O ₁₄ S ₄	707.0510	705.0368	707.0524	705.0379	-1.98/-1.56
VIII	C ₁₈ H ₂₀ N ₆ O ₉ S ₃	561.0524	559.0363	561.0527	559.0381	-0.53/-3.22
IX	C ₁₈ H ₂₄ N ₈ O ₁₃ S ₄	689.0446	687.0286	689.0436	687.0277	1.45/1.31
X	C ₃₀ H ₃₂ N ₁₄ O ₁₁ S ₅	925.1018	923.0922	925.1013	923.0906	0.54/1.73
XI	C ₁₄ H ₁₅ N ₅ O ₆ S ₂	414.0524	412.0410	414.0537	412.0391	-3.14/4.61

^a The data of theoretical $[M+H]^+$ and $[M-H]^-$ was calculated by software of Shimadzu accurate mass calculator.

dimension RPLC-UV chromatograms of peak 2 in Fig. 1 of different LC methods. Compared with the chromatograms of methods A and B, method C which used ammonium formate solution (10 mM) and ammonium formate (8 mM) in [acetonitrile-water (4:1, v/v)] solution as the mobile phase in the second dimension system could achieve better separation.

3.2. 2D LC Separation and mass spectra results of impurities

Fig. 2 showed the gel chromatography of cefodizime sodium(A), cefonicid sodium(B) and cefmenoxime hydrochloride(C) in the first dimension, there were six polymerized peaks before the main peaks could be separated by the chromatographic condition in the first dimension system with the detection wavelength 254 nm (peaks 1–6). To check whether each of the impurity peaks was pure or not,

it was important to confirm that all the impurities eluting before the main peaks. The effluent of each peak (peaks 1–6) on the gel chromatographic system before the main peak was switched to the second dimension chromatographic system to obtain RPLC-UV chromatogram of six polymer peaks (seen the insert figure in Fig. 2) and analyzed by LC-MS. There were eleven impurities could be separated in the second dimension and the structures of eleven products were deduced based on the high-resolution MSⁿ data, in which nine impurities were polymerized impurities. Table 1 showed eleven impurities of cefodizime, cefmenoxime and cefonicid in the positive and negative ion modes, and the values of deviation were basically less than 5 ppm. Table 2 showed ESI-MSⁿ exact mass data of major product ions of eleven impurities in both positive and negative ion modes, and proposed chemical structures

Table 2
ESI–MSⁿ exact mass data of major product ions of cefodizime, cefmenoxime and cefonicid and eleven high-molecule weight impurities in positive and negative ion modes.

Imp.	[M+H] ⁺ (<i>m/z</i>)	MS ² fragmentation ions (<i>m/z</i>)	MS ³ fragmentation ions (<i>m/z</i>)
I	998.0796	585.0261 980.0520, 954.0783, 414.0532	396.0461
II	797.0620	779.0353, 608.0648	
III	731.0367	542.0529, 498.0583, 470.0583, 220.0908	
IV	980.0700	791.0709, 730.0544, 636.0644	
V	968.0405	779.0442, 718.0277, 396.0365	
VI	1026.1127	585.0280 422.0822	396.0451
VII	707.0510	663.0490, 497.9773, 470.9738	
	705.0368	661.0480, 617.0567	
VIII	561.0524	365.0750	
	559.0363	487.0454	423.0747, 379.0866, 345.1009
IX	689.0446	423.0842, 379.0938, 345.1034, 327.0921	581.0544
	671.0256		
	645.0511, 627.0299, 581.0595		
	687.0286	643.0463, 599.0551	
X	925.1018	881.0969, 809.0832, 765.0880, 748.0833, 704.0694	
XI	414.0515	396.0429, 370.0633, 352.0544, 326.0746, 169.0486	
Imp.	[M–H] [–] (<i>m/z</i>)	MS ² fragmentation ions (<i>m/z</i>)	MS ³ fragmentation ions (<i>m/z</i>)
VII	705.0368	661.0480, 617.0567	
VIII	559.0363	487.0454	423.0747, 379.0866, 345.1009
IX	687.0286	423.0842, 379.0938, 345.1034, 327.0921	
		643.0463, 599.0551	

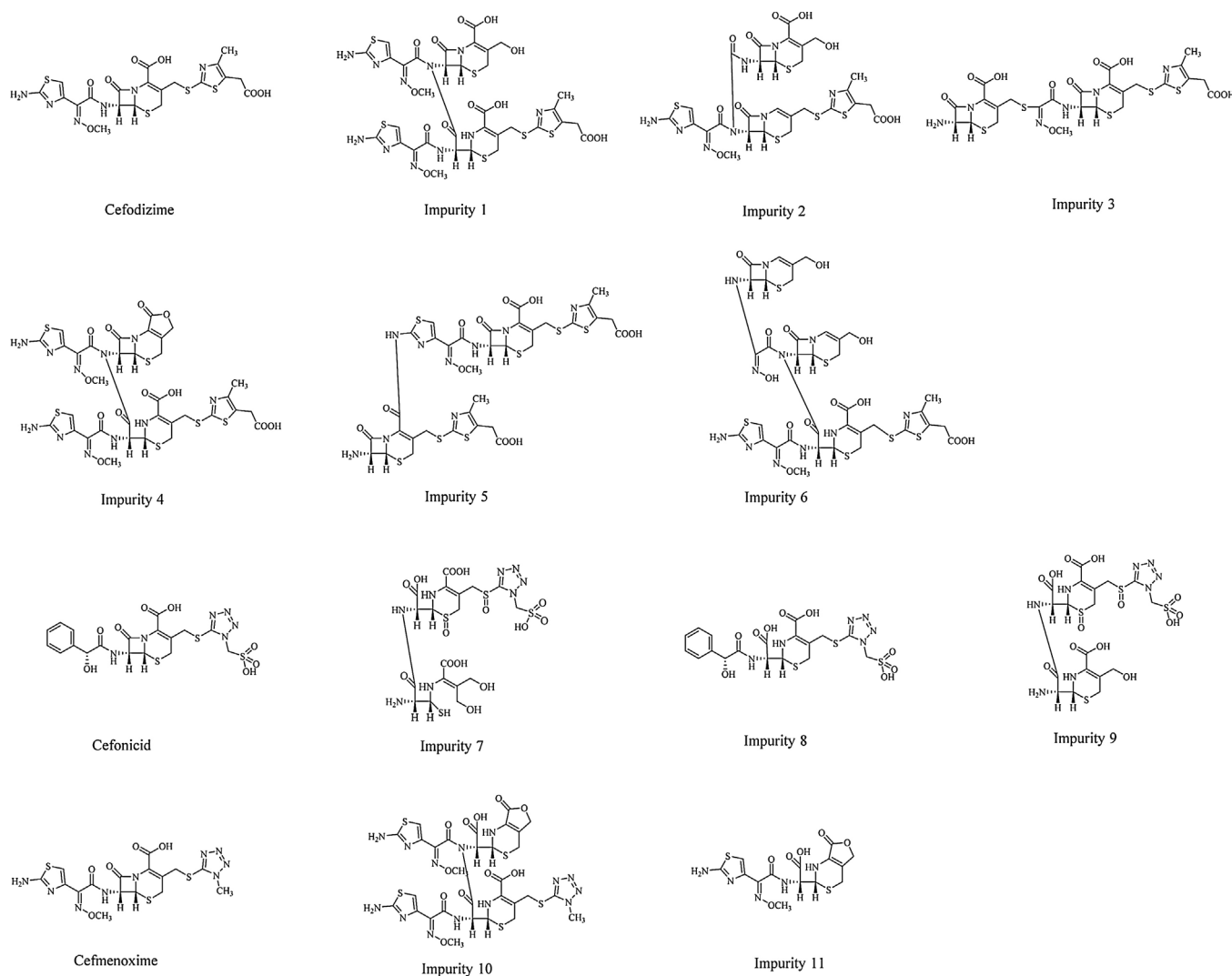


Fig. 3. Chemical structures of cefodizime, cefonicid and cefmenoxime and impurities 1–11.

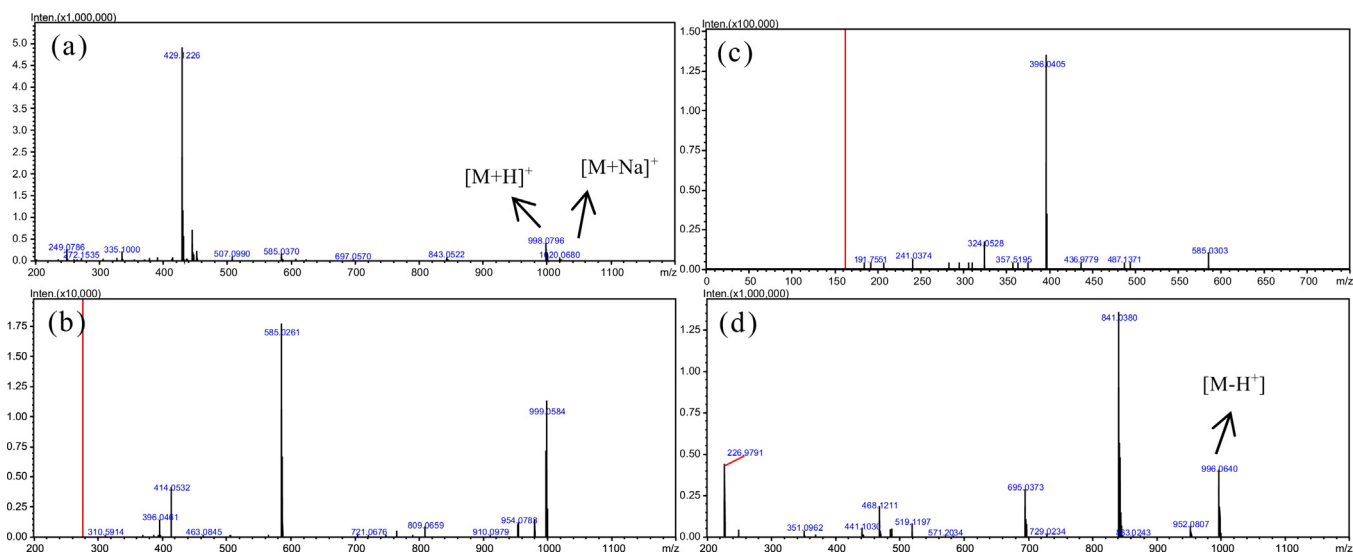


Fig. 4. (a) Full scan spectrum of impurity 1 showing $[M+H]^+$ ions at m/z 998.0796. (b) MS^2 spectrum of m/z 998.0796 ion. (c) MS^3 spectrum of m/z 585.0261 ion. (d) Full scan spectrum of impurity 1 showing $[M-H]^-$ ions at m/z 996.0640.

of cefodizime, cefmenoxime and cefonicid and eleven impurities were shown in Fig. 3.

3.3. Structure elucidation

3.3.1. Impurity I

TOF high resolution mass showed that the formula of impurity I was $C_{34}H_{35}N_{11}O_{13}S_6$. Fig. 4 revealed that $[M+H]^+$ at m/z 998.0796 and $[M+Na]^+$ at m/z 1020.0680 appeared in the full scan of ESI+. The protonated molecule at m/z 998.0796 of impurity I fragmented into the product ion at m/z 954.0783 by means of the loss of 44 Da, corresponding to CO_2 , further fragmented into the product ion at m/z 585.0261 by the cleavage of amide bond between C-18 and N-29 and loss of $C_{14}H_{15}N_5O_4S_2$ structure. Subsequent loss of one 2-mercapto-4-methyl-5-thiazoleacetic acid (MMTA) led to the ion at m/z 396.0461. And the ion at m/z 980.0520 was formed by elimination of one H_2O . The protonated molecule at m/z 998.0796 showed the cleavage of amide bond between C-18 and N-29 and loss of $C_{20}H_{20}N_6O_7S_4$ structure conducted to the fragment ion at m/z 414.0532. From the above information, it was concluded that impurity I was resulting from intermolecular aminolysis of β -lactam ring by the amine of the second molecule with the hydroxyl substituting MMTA [15]. Fig. 5 presented the proposed structure and fragmentation pattern of impurity I.

3.3.2. Impurity II

TOF high resolution mass showed that the formula of impurity II was $C_{28}H_{28}N_8O_{10}S_5$. The protonated molecule at m/z 797.0620 of impurity II fragmented into the product ion at m/z 608.0648 by means of the loss of 189 Da, corresponding to MMTA. Another fragmentation pathway of the protonated molecule at m/z 797.0620 concerned the loss of 18 Da, corresponding to one H_2O , conducting to the fragment ion at m/z 779.0353. These experiments revealed that structure of impurity II was formed by intermolecular aminolysis at the position of C-29 by the amine of the second molecule with the hydroxyl substituting MMTA. Fig. S2 presented the proposed structure and fragmentation pattern of impurity II.

3.3.3. Impurity III

TOF high resolution mass showed that the formula of impurity III was $C_{28}H_{27}N_8O_{10}S_5$. The protonated molecule at m/z 731.0367 of impurity III fragmented into the product ion at m/z 542.0592 by

loss of one MMTA, further fragmented into the ion at m/z 498.0583 by the loss of one CO_2 and the ion at m/z 220.0908 by elimination of $C_7H_6N_2O_3S_2$ structure and one H_2S . Another fragmentation pathway of the ion at m/z 542.0529 concerned the loss of one CO_2 and one CO , conducting to the fragment ion at m/z 470.0583. From the above information, the structure of impurity III was deduced that the carbon positive ion formed by the bond cleavage at the position of C-10 in one molecule of cefodizime, and the carbon positive ion could nucleophilic attack of the atom at the S-25 of another molecule with the cleavage of amide bond. Fig. S3 presented the proposed structure and fragmentation pattern of impurity III.

3.3.4. Impurity IV

The formula of impurity IV was $C_{34}H_{33}N_{11}O_{12}S_6$ based on the data from TOF high resolution mass spectrometer. The fragmentation pathway of the protonated molecule at m/z 980.0700 fragmented into the product ion at m/z 791.0709 by the loss of one MMTA, further fragmented into the ion at m/z 730.0544 by elimination of one NH_3 and one CO_2 . Another fragmentation pathway of ion at m/z 791.0709 concerned the loss of $C_3H_2N_2S_2$ structure, leading to the ion at m/z 636.0644. From the above information, it was concluded that impurity IV was resulting from intermolecular aminolysis of β -lactam ring by the amine of the second molecule with losing one MMTA and forming a five-member lactone ring. Fig. S4 presented the proposed structure and fragmentation pattern of impurity IV.

3.3.5. Impurity V

TOF high resolution mass showed that the formula of impurity V was $C_{34}H_{33}N_9O_{11}S_7$. The protonated molecule at m/z 968.0405 fragmented into the product ion at m/z 779.0442 by the loss of one MMTA, further fragmented into the ion at m/z 718.0277 by loss of one H_2O and one NH_3 . The product ion at m/z 779.0442 showed the cleavage of the amide bond between N-15 and C-30 conducted to the ion at m/z 396.0365. Based on the information above, the structure of impurity V was that the carboxyl group at the position of C-30 of one molecule cefodizime reacted with the amino group at the position of N-15 of the second molecule to form an amide bond accompanied with amide bond cleavage at the position of N-29. Fig. S5 presented the proposed structure and fragmentation pattern of impurity V.

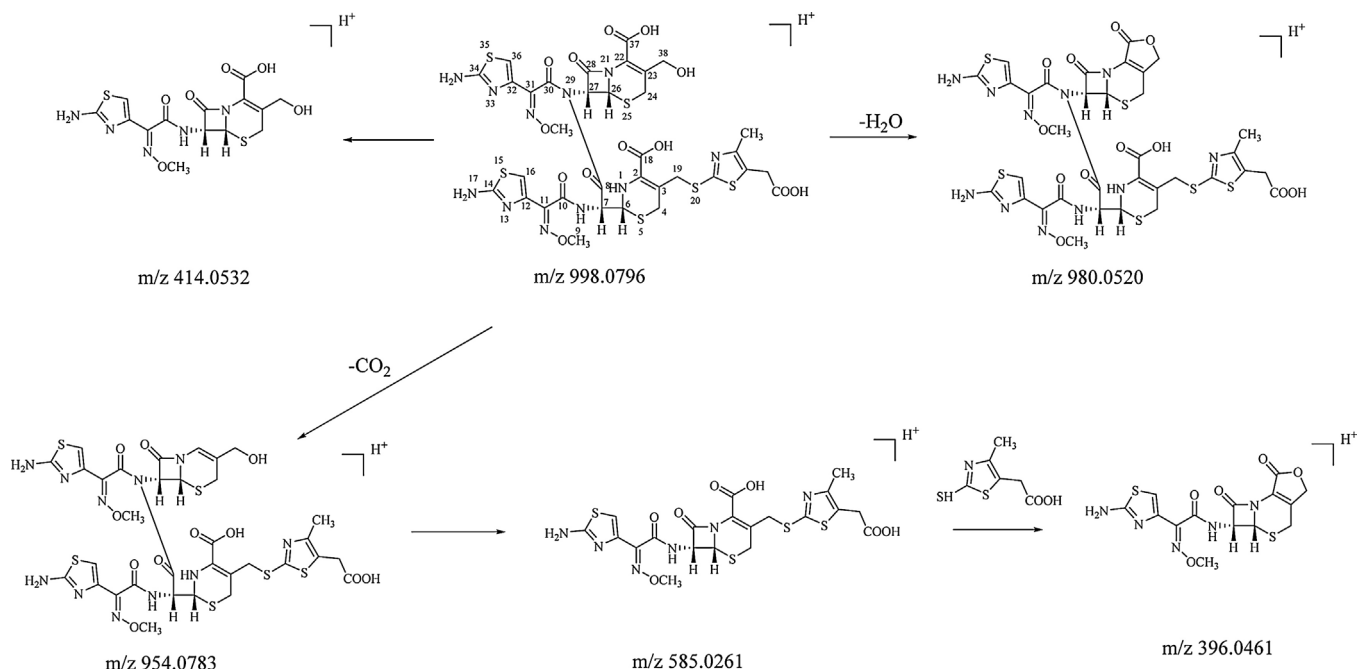


Fig. 5. proposed fragmentation pathways of impurity I in the positive ion mode.

3.3.6. Impurity VI

High resolution TOF mass scanning in both positive and negative modes proved that the formula of impurity VI was $C_{36}H_{39}N_{11}O_{13}S_6$. The protonated molecule at m/z 1026.1127 of impurity VI fragmented into the product ion at m/z 585.0280 and the ion at m/z 442.0822 by cleavage of amide bond between C-8 and N-29. The product ion at m/z 585.0280 fragmented into the ion at m/z 396.0451 by the loss of one MMTA. From the above information, the structure of impurity VI was that intermolecular aminolysis of β -lactam ring by the amine of the second molecule with the hydroxyl substituting MMTA. Subsequently, the carbon positive ion formed by the bond cleavage at the position of C-31, and the carbon positive ion could nucleophilic attack of the amine at the position of N-41 of the third molecule with the cleavage of amide bond and hydroxyl substituting MMTA. Fig. S6 showed the mass spectral fragmentation of impurity VI in both modes.

3.3.7. Impurity VII

Data obtained from TOF high resolution mass spectrometer in both positive and negative ion mode showed that the formula of impurity VII was $C_{18}H_{26}N_8O_{14}S_4$. The protonated molecule at m/z 707.0510 fragmented into the product ion at m/z 663.0490 by losing one CO_2 and the ion at m/z 497.9773, which was easy to be produced, by the cleavage of the bond between N-1 and C-6 and elimination of $C_5H_9NO_4$ structure. The fragment ion at m/z 470.9738, which originated from the loss of one molecule of NH_3 from the ion at m/z 497.9773. In the negative ion mode, the product ion at m/z 661.0480, which originated from the loss of one molecule of CO_2 from the precursor ion at m/z 705.0368, further fragmented into the product ion at m/z 617.0567 by the loss of one CO_2 . Based on the information above, the structure of impurity VII was that intermolecular aminolysis of β -lactam ring by the amine of the second molecule, and one molecule in the structure of impurity VII was the cleavage of amide bond of cefonicid with oxygen addition occurred on the sulphurs, and the other one was the cleavage of amide bond of cefonicid accompanied with hydroxyl substituting 5-Mercapto-1-methyltetrazole (3-TSA) and the cephem ring was hydrolyzed.

Figs. S7 and S8 presented the proposed structure and fragmentation pathway of impurity VII.

3.3.8. Impurity VIII

The structure of impurity VIII was $C_{18}H_{20}N_6O_9S_3$ based on the data from TOF high resolution mass spectrometer. The fragmentation pathway from the protonated molecule at m/z 561.0524 concerned the loss of 3-TSA leading to the ion at m/z 365.0750. In the negative ion mode, the precursor ion at m/z 559.0363 fragmented into the product ion at m/z 487.0454 by losing one CO and one CO_2 and the ion at m/z 423.0842 by elimination of one SO_2 , further loss of one CO_2 to form the ion at m/z 379.0938. The fragment ion at m/z 345.1034, which originated from the loss of one molecule of H_2S from the ion at m/z 379.0938, further fragmented into the product ion at m/z 327.0921 by the loss of one H_2O . The impurity VIII was a hydrolysis product of cefonicid, the structure of impurity VIII was deduced as the hydrolysis of β -lactam ring of cefonicid. Figs. S9 and S10 presented the proposed structure and fragmentation pattern of impurity VIII.

3.3.9. Impurity IX

The formula of impurity IX was $C_{18}H_{24}N_8O_{13}S_4$ based on the data from TOF high resolution mass spectrometer. The protonated molecule at m/z 689.0446 of impurity IX fragmented into the product ion at m/z 671.0256 by means of the loss of one H_2O . Subsequent loss of one CO and one H_2O conducted to the fragment ion at m/z 581.0595. Another fragmentation pathway of the protonated molecule at m/z 689.0446 fragmented into the product ion at m/z 645.0511 by losing one CO_2 and the ion at m/z 627.0299 by elimination of one H_2O . In the negative ion mode, the precursor ion at m/z 687.0286 fragmented into the product ion at m/z 643.0463 by the loss of one CO_2 and the ion at m/z 599.0551 by elimination of one CO_2 . From the above information, the structure of impurity IX was that intermolecular aminolysis of β -lactam ring by the amine of the second molecule, and one molecule was the cleavage of amide bond of cefonicid with oxygen addition occurred on the sulphurs, and the other one was the cleavage of the amide bond of cefonicid accompanied with hydroxyl substituting 3-TSA. Figs. S11 and

S12 presented the proposed structure and fragmentation pattern of impurity IX.

3.3.10. Impurity X

The formula of impurity X was $C_{30}H_{32}N_{14}O_{11}S_5$ based on the data from TOF high resolution mass spectrometer. The fragmentation pathway from the protonated molecule at m/z 925.1018 concerned the loss of one 5-mercapto-1-methyltetrazole leading to the ion at m/z 809.0832. Subsequent loss of one CO_2 conducted to the ion at m/z 765.0880. The product ion at m/z 765.0880 fragmented into the ion at m/z 748.0833 by the loss of one NH_3 and the ion at m/z 704.0694 by the elimination of one CO_2 . Another fragmentation pathway of the ion at m/z 925.1018 concerned the loss of one CO_2 , leading to the ion at m/z 881.0969. From the above information, the structure of impurity X was formed by intermolecular aminolysis of β -lactam ring by the amine of the second molecule with losing one 5-mercapto-1-methyltetrazole and forming a five-member lactone ring and the β -lactam ring was hydrolyzed. Fig. S13 presented the proposed structure and fragmentation pattern of impurity X.

3.3.11. Impurity XI

Data obtained from TOF high resolution mass spectrometer in both positive and negative ion mode shows that the formula of impurity XI was $C_{14}H_{15}N_5O_6S_2$. The protonated molecule at m/z 414.0515 fragmented into the product ion at m/z 396.0429 by the loss of one H_2O and the ion at m/z 352.0544 by elimination of one CO_2 , subsequent loss of one $C_6H_5N_2O_2S$ structure leading to the ion at m/z 169.0486. Another fragmentation pathway of the protonated molecule at m/z 414.0515 concerned the loss of 44 Da, corresponding to one CO_2 , conducting to the fragment ion at m/z 370.0633, further loss of one CO_2 conducted to the ion at m/z 326.0746. From the above information, impurity XI was formed by losing one 5-mercapto-1-methyltetrazole with forming a five-member lactone ring and the β -lactam ring of cefmenoxime was hydrolyzed. Impurity XI was a hydrolysis product of cefpiramide. Fig. S14 presented the proposed structure and fragmentation pattern of impurity X.

3.4. Forming mechanism of β -lactam antibiotic polymerization in cephalosporins

β -lactam antibiotic polymerization usually could be divided into two categories: 1) occurred in the nucleus of the N-type polymerization: First, the β -lactam ring was opened to form a secondary amino group with nucleophilic attack ability, then nucleophilic addition reaction happened to the carbonyl group of the other cephalosporin molecule, and the N-type polymerization was formed such as impurities I and VI; 2) side-chain involvement in the L-type polymerization: The active group in the 7-side chain directly attacked the carbonyl carbon atoms in the β -lactam ring to form a polymer such as impurity V [16].

Moreover, β -lactam antibiotics included ring-closed and ring-open polymer [17]. The polymerization process could be seen from the impurity I–X. It could be seen that the β -lactam ring-open was the structural basis of the polymerization. It was found that the energy of the β -lactam ring and amide conjugation can determine the molecular reactivity. The amide nitrogen atoms of the β -lactam ring generally extended out of a certain length of the plane composed of three adjacent carbon atoms to form the taper of the β -lactam ring. When the taper of the ring increased, the tension energy and the conjugate energy of the ring were decreased, the reaction activity of the molecule was increased, and the opening ring became easy [18].

4. Conclusions

Eleven allergic impurities in cefodizime, cefmenoxime and cefonicid were separated and characterized by a trap-free 2D-HPSEC $\times$ LC-IT-TOF MS with positive and negative modes of electrospray ionization method. Through the multidimensional heart-cutting approaches and online demineralization technique, the contradiction between non-volatile mobile phase and mass spectrometry was solved. The TIC chromatogram of LC-MS could be in conformity with the LC chromatogram of the official analytical method in the peak sequence of impurities. Moreover, an effective method to characterize the polymerized impurities in β -lactam antibiotics may be established on the basis of column-switching technique which effectively combined the advantages of HPSEC and the ability of RP-HPLC to identify the special impurities. Structures of eleven impurities in cephalosporins were deduced based on the high resolution MSⁿ data with both positive and negative modes, in which nine impurities were polymerized impurities. And the forming mechanism of β -lactam antibiotic polymerization in cephalosporins was also studied.

Acknowledgment

This research is supported by the food and drug science and technology project of Zhejiang food and drug administration in 2016.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jpba.2017.07.063>.

References

- [1] C.Y. Lu, C.H. Feng, Identification of dimer impurities in ampicillin and amoxicillin by capillary LC and tandem mass spectrometry, *J. Sep. Sci.* 30 (2007) 329–332.
- [2] S.Y. Cai, C.Q. Hu, M.Z. Xu, Chromatographic determination of high-molecular weight impurities in amoxicillin, *J. Pharm. Biomed. Anal.* 31 (2003) 589–596.
- [3] Q. Zhou, R. Ding, W.H. Zhou, Process development on the determination of high molecular weight impurities in cephalosporin, *Chin. J. Antibiot.* 27 (2002) 181–182.
- [4] Y.R. Hou, Y.Z. Yuan, M. Zhang, W. Qian, T.J. Hang, Determination of high molecular mass impurities in cefuroxime axetil and its preparation by polystyrene-based gel filtration chromatography, *Chin. J. Pharm. Anal.* 32 (2012) 267–272.
- [5] H. Ruan, D.D. Chen, Y.X. Jin, Y. Chen, Determination of high molecular polymers in cefoperazone sodium and sulbactam sodium for injection by molecular exclusion chromatography, *Chin. J. Mod. Appl. Pharm.* 30 (2013) 79–83.
- [6] Y.N. Zhong, J.H. Liu, X.J. Lin, X.C. Li, HPLC determination of high molecular mass impurities in ticarcillin disodium and clavulanate potassium (15:1), *Chin. J. Pharm. Anal.* 31 (2011) 871–874.
- [7] S.S. Deng, K.H. Tang, M.Q. Xu, Y.C. Wang, C.R. Zhang, J.X. Zhang, Z.Y. Li, Determining the polymers in ceftazidime sodium by HPSEC and its structure identification, *Chin. J. Antibiot.* 41 (2016) 227–231.
- [8] Y.Y. Wang, Y. Chen, L.Y. Hong, Review on progresses of isolation and test methods for high-molecular weight polymer impurities in β -lactam antibiotics, *Chin. Pharm. Aff.* 29 (2015) 608–611.
- [9] S. Horimoto, T. Mayumi, K. Aoe, N. Nishimura, T. Sato, Analysis of β -lactam antibiotics by high performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry using bromoform, *J. Pharm. Biomed. Anal.* 30 (2002) 1093–1102.
- [10] D.T. Modhave, T. Handa, R.P. Shah, S. Singh, Stress degradation studies on lornoxicam using LC: LC-MS/TOF and LC-MSⁿ, *J. Pharm. Biomed. Anal.* 56 (2011) 538–545.
- [11] J. Wang, F. Gao, H. Wang, W. Su, Characterization of the oxidation degradation Products in tigecycline by column-switching and online demineralization technique for dual gradient liquid chromatography combined With Q orbitrap mass spectrometry, *Chromatographia* 79 (2016) 537–545.
- [12] Y. Zhang, L. Zeng, C. Pham, R. Xu, Preparative two-dimensional liquid chromatography/mass spectrometry for the purification of complex pharmaceutical samples, *J. Chromatogr. A* 1324 (2014) 86–95.
- [13] J. Wang, F. Gao, H. Wang, W. Su, Characterization of the oxidation degradation Products in tigecycline by column-switching and online demineralization

- technique for dual gradient liquid chromatography combined With Q orbitrap mass spectrometry, *Chromatographia* 79 (2016) 537–545.
- [14] J.L. Zhang, Y. Zhang, S.Q. Zhao, J.T. Yao, M. Zhang, Y.Z. Yuan, Structure identification of related substances in doxycycline hyclate by 2D-LC-IT-TOF/MS, *Chin. Pharm. J.* 50 (2015) 2073–2080.
- [15] S.Y. Cai, C.Q. Hu, Chromatographic determination of polymerized impurities in meropenem, *J. Pharm. Biomed. Anal.* 37 (2005) 585–589.
- [16] X.L. Jiang, K. Liu, J.F. Deng, B. Li, Research progress in the high molecular weight impurities of cephalosporins, *World Notes Antibiot.* 28 (2007) 264–269.
- [17] M. Xia, T.J. Hang, X.M. Li, P. Zhang, A review on the test methods for high-molecular weight polymer impurities in β -lactam antibiotics, *Prog. Pharm. Sci.* 31 (2007) 501–506.
- [18] X.Y. Zhang, T. Wang, C.Q. Li, Z.L. Chen, Research progress of penicillin antibiotic polymeric polymer, *Cent. South Pharm.* 9 (2011) 520–524.

Supporting information

Yu Xu, DanDan Wang, Lan Tang, Jian Wang

Figure S1 The second dimension RPLC-UV chromatograms of peak 2 in figure 1 of different LC methods.

Figure S2 proposed fragmentation pathways of impurity in the positive ion mode.

Figure S3 proposed fragmentation pathways of impurity in the positive ion mode.

Figure S4 proposed fragmentation pathways of impurity in the positive ion mode.

Figure S5 proposed fragmentation pathways of impurity in the negative ion mode.

Figure S6 proposed fragmentation pathways of impurity in the positive ion mode.

Figure S7 proposed fragmentation pathways of impurity in the positive ion mode.

Figure S8 proposed fragmentation pathways of impurity in the negative ion mode.

Figure S9 proposed fragmentation pathways of impurity in the positive ion mode.

Figure S10 proposed fragmentation pathways of impurity in the negative ion mode.

Figure S11 proposed fragmentation pathways of impurity in the positive ion mode.

Figure S12 proposed fragmentation pathways of impurity in the negative ion mode.

Figure S13 proposed fragmentation pathways of impurity in the positive ion mode.

Figure S14 proposed fragmentation pathways of impurity in the positive ion mode.

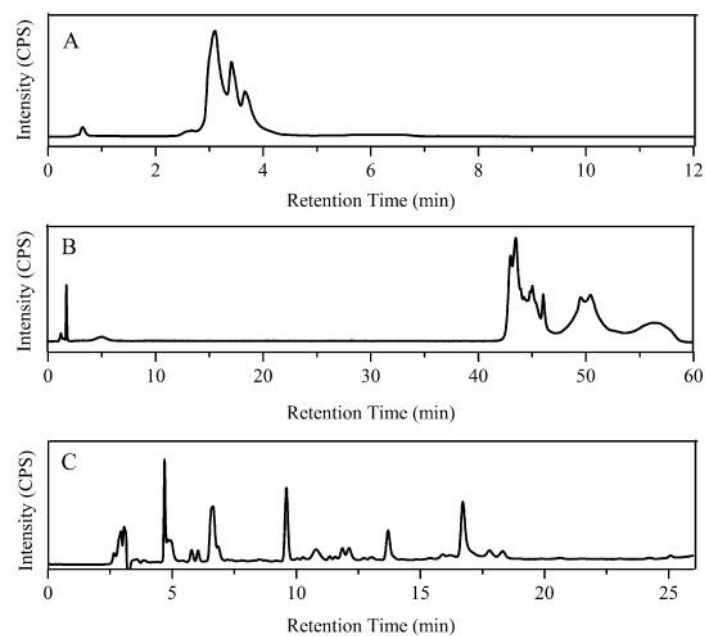


Figure S1 The second dimension RPLC-UV chromatograms of peak 2 in figure 1 of different LC methods.

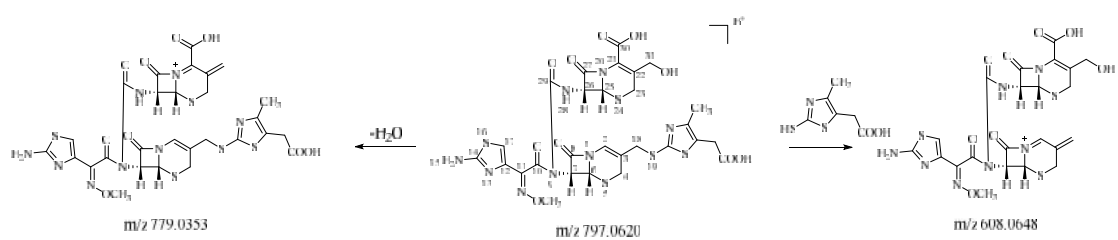


Figure S2 proposed fragmentation pathways of impurity in the positive ion mode.

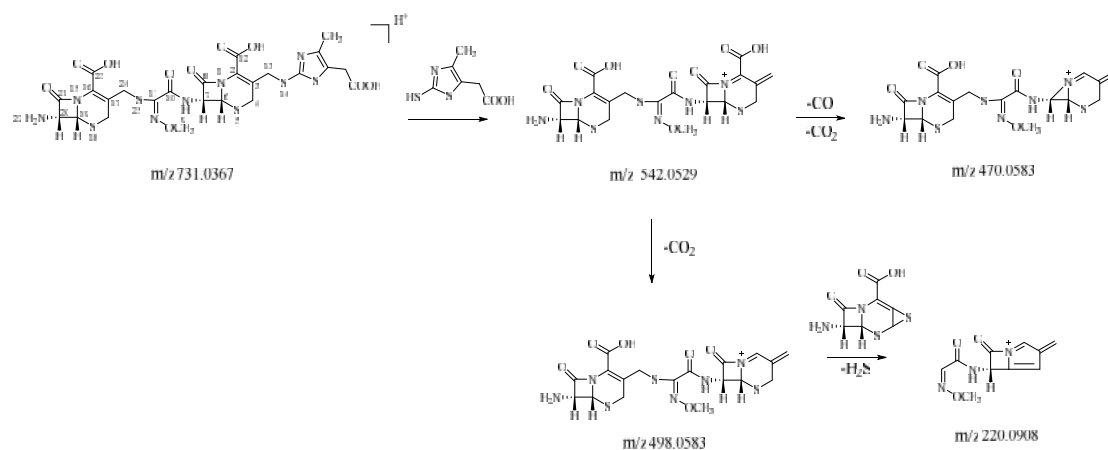


Figure S3 proposed fragmentation pathways of impurity in the positive ion mode.

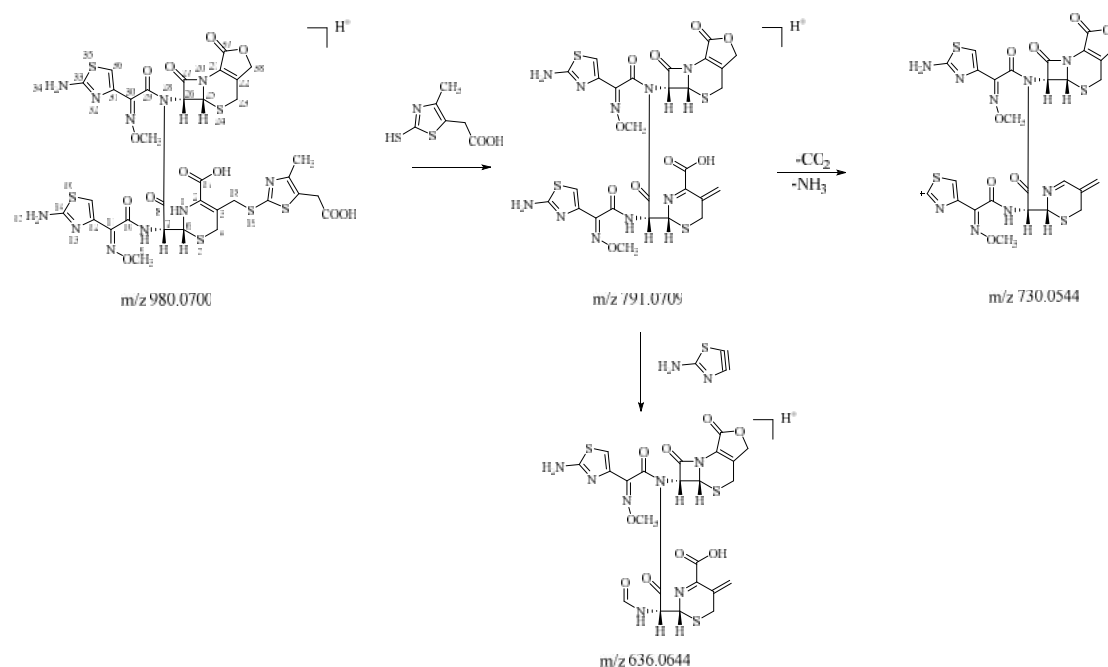


Figure S4 proposed fragmentation pathways of impurity in the positive ion mode.

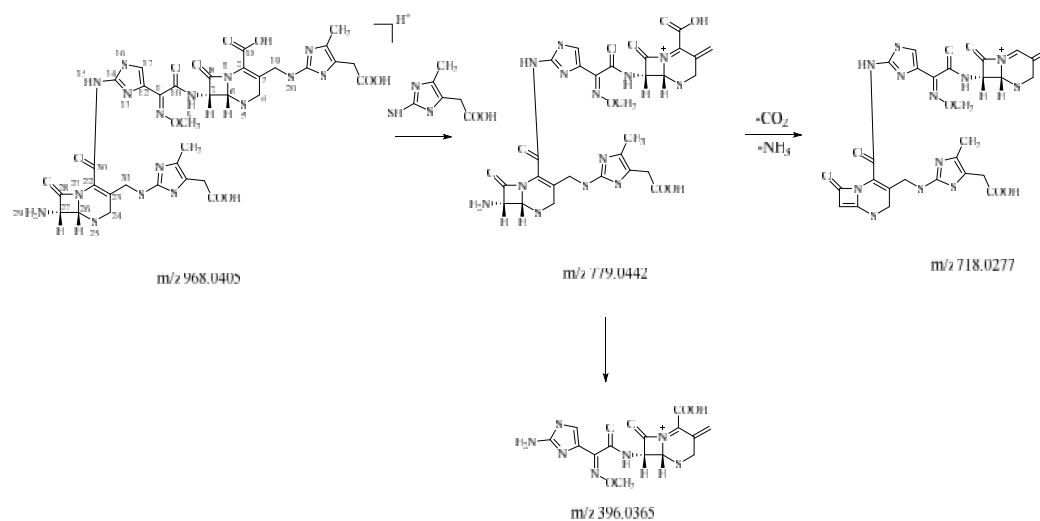


Figure S5 proposed fragmentation pathways of impurity in the negative ion mode.

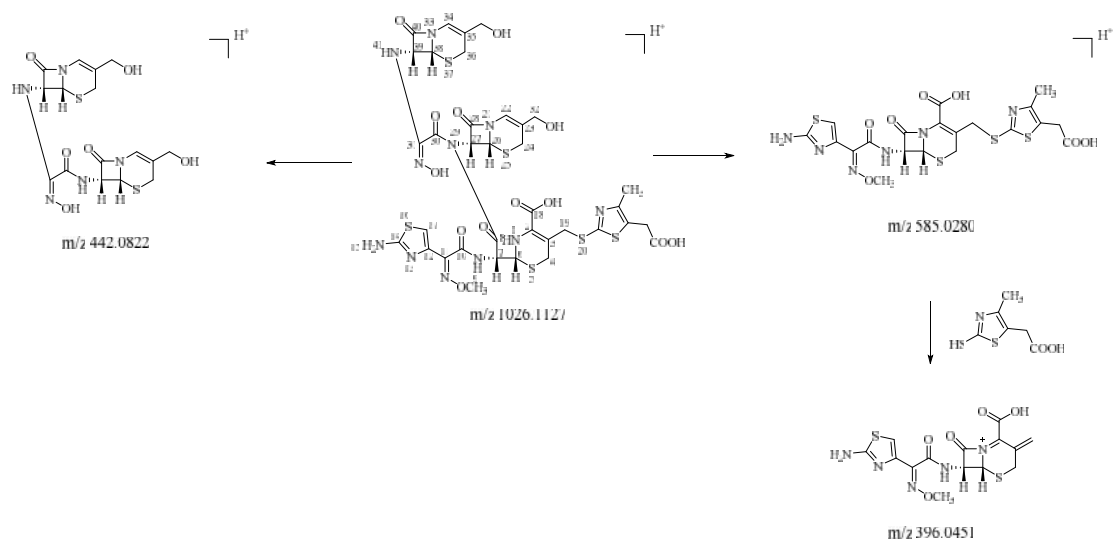


Figure S6 proposed fragmentation pathways of impurity in the positive ion mode.

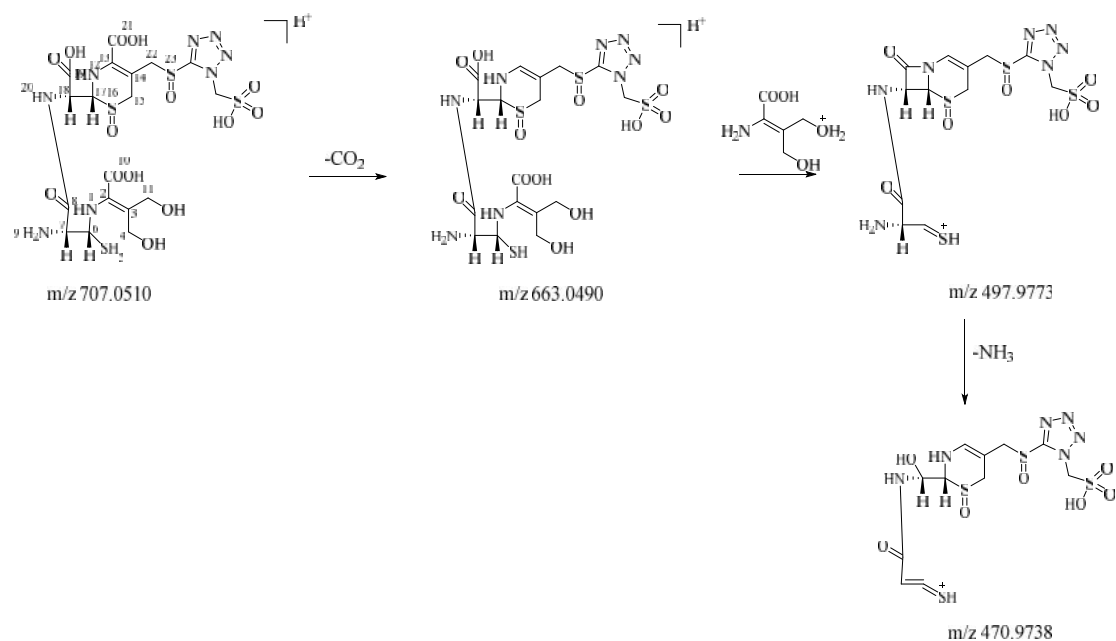


Figure S7 proposed fragmentation pathways of impurity in the positive ion mode.

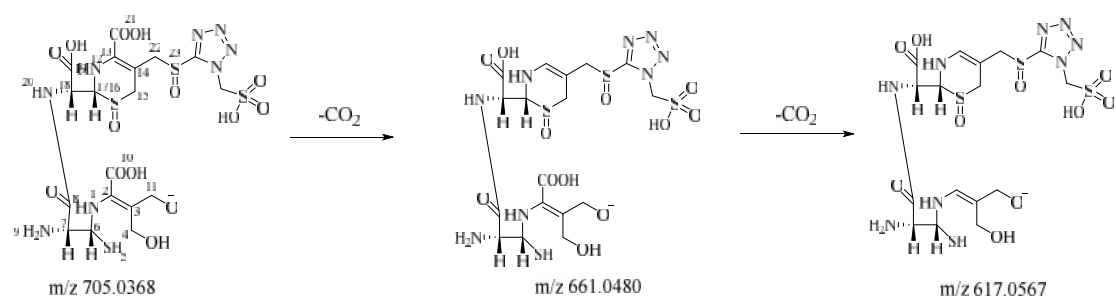


Figure S8 proposed fragmentation pathways of impurity in the negative ion mode.

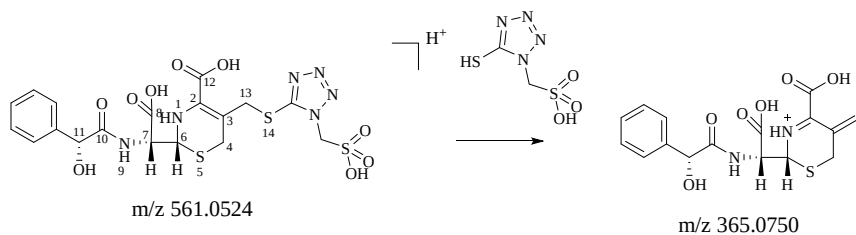


Figure S9 proposed fragmentation pathways of impurity in the positive ion mode.

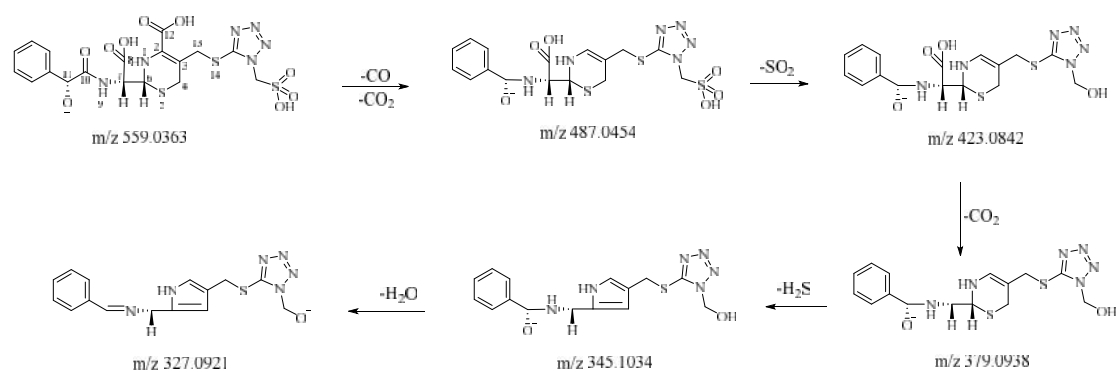


Figure S10 proposed fragmentation pathways of impurity in the negative ion mode.

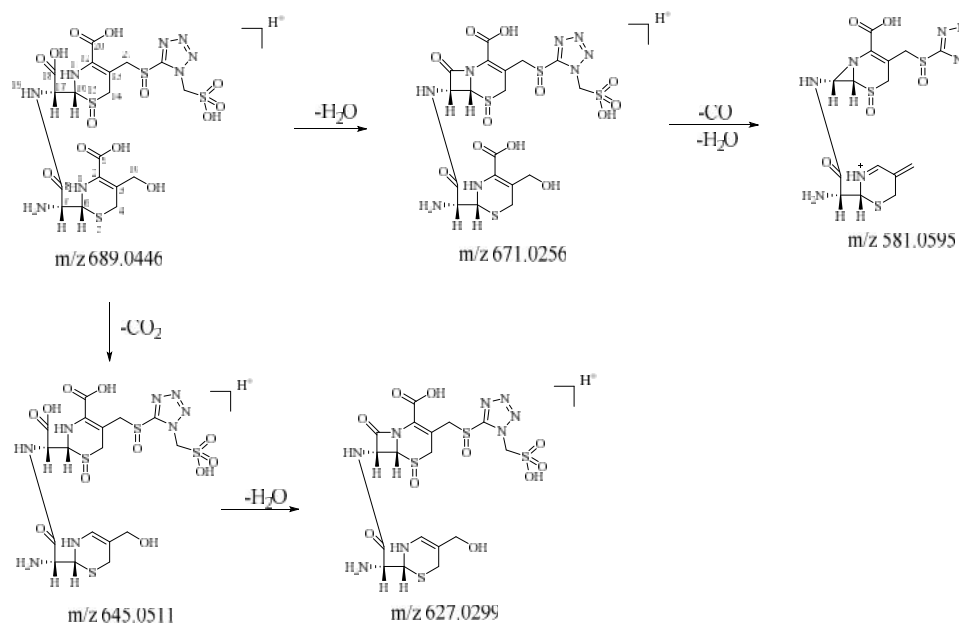


Figure S11 proposed fragmentation pathways of impurity in the positive ion mode.

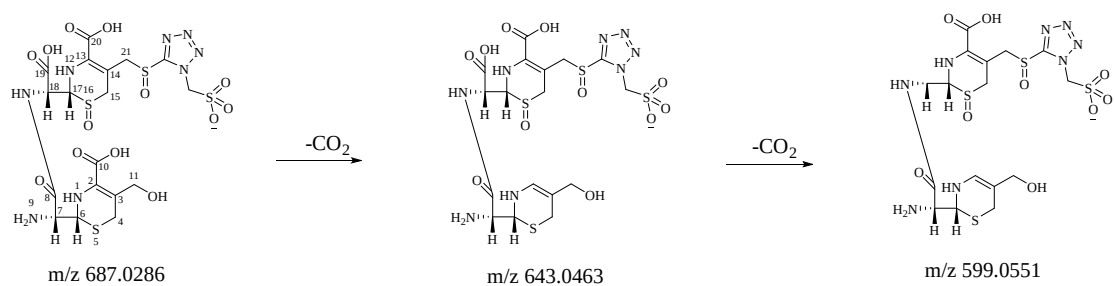


Figure S12 proposed fragmentation pathways of impurity in the negative ion mode.

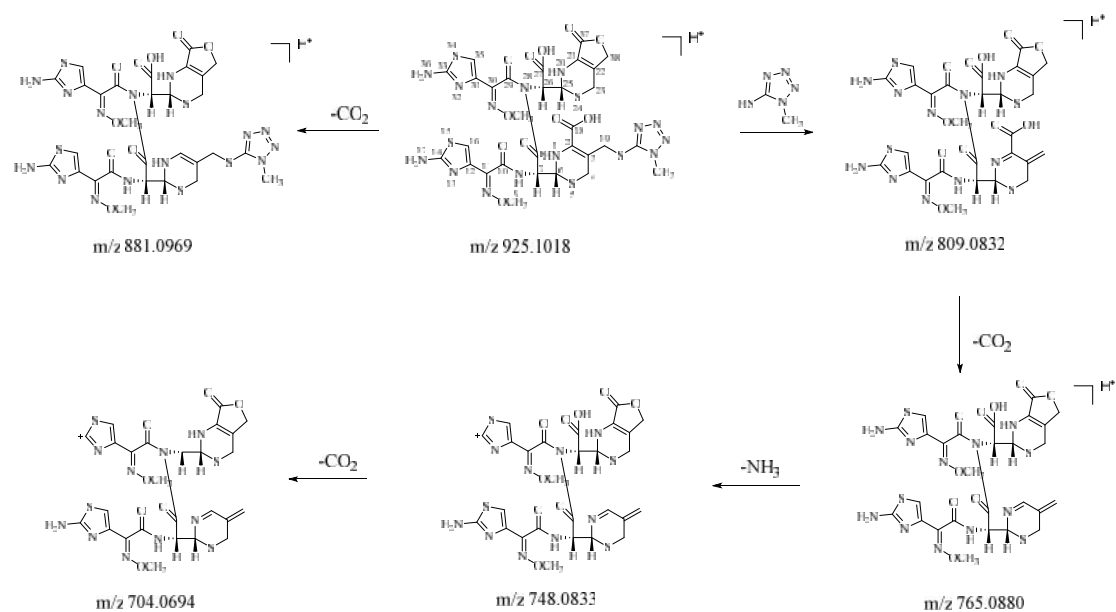


Figure S13 proposed fragmentation pathways of impurity in the positive ion mode.

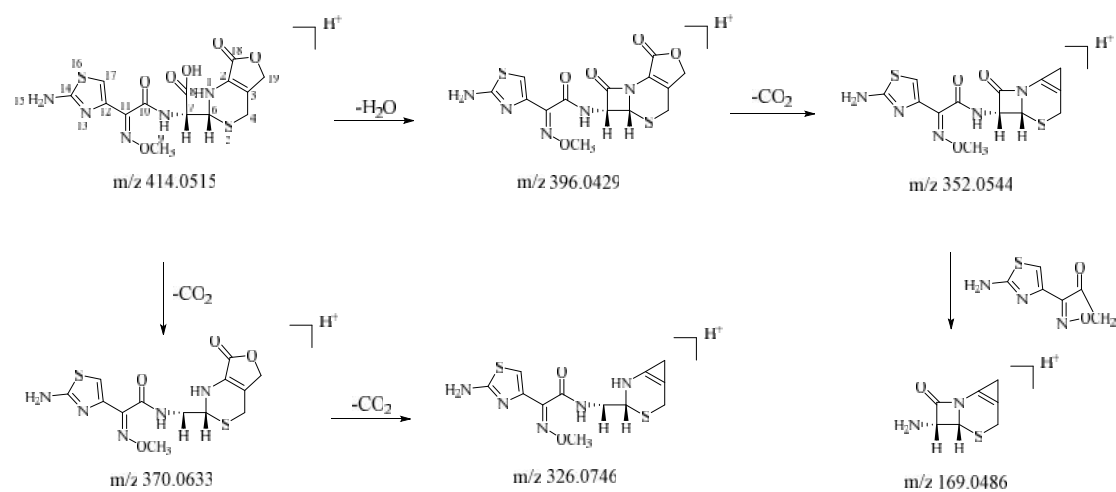


Figure S14 proposed fragmentation pathways of impurity in the positive ion mode.