



Separation and characterization of allergenic polymerized impurities from cephalosporin for injection by trap free two-dimensional high performance size exclusion chromatography $\times$ reversed phase liquid chromatography coupled with ion trap time-of-flight mass spectrometry

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ARTICLE INFO

Article history:

Received 1 February 2018

Received in revised form 18 March 2018

Accepted 19 March 2018

Available online 20 March 2018

Keywords:

Cephalosporin for injection

Allergenic polymerized impurity

High performance size exclusion

chromatography

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

ABSTRACT

As requested by regulatory authorities, polymerized impurities are an important issue of quality control. In this study, we presented the utilization of a trap-free two-dimensional chromatography, which was consisted by a high performance size exclusion chromatography (HPSEC) and a reversed-phase liquid chromatography (RP-LC) coupled to ion trap time-of-flight mass spectrometry with positive mode of electrospray ionization, to separate and characterize ten allergenic impurities in ceftazidime for injection, cefazolin sodium for injection, cefoperazone sodium and sulbactam sodium for injection and cefamandole nafate for injection. An effective method for characterizing the polymerized impurities in β -lactam antibiotics was 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. In the first dimension, the column was the Xtimate SEC-120 analytical column (7.8 mm $\times$ 30 cm, 5 μ m) and the flow rate was 1.0 mL min⁻¹ with gradient elution using 0.005 mol L⁻¹ phosphate buffer solution at pH 7.0 and acetonitrile as mobile phase. In the second dimension, the analytical column was ZORBAX SB-C18 (4.6 $\times$ 150 mm, 3.5 μ m) using ammonium formate solution (10 mM) and ammonium formate (8 mM) in [acetonitrile-water (4:1, v/v)] solution as mobile phase at a flow rate of 0.4 mL min⁻¹. Eluent associate with each peak separated in the first dimension was trapped by a 20 μ L quantitative loop and then transferred (via a six-port valve) into the second dimension system with volatile mobile phase. Through the multiple heart-cutting 2D-LC approach and online desalting technique, the problem of incompatibility between non-volatile mobile phase and mass spectrometry was solved completely. The fragmentation behaviors of ten allergenic impurities were studied. The structures of ten allergenic impurities in cephalosporin drug substance were deduced based on the HPLC-MSⁿ data, in which four impurities were polymerized impurities. The forming factors of polymerized impurity in cephalosporins were also studied.

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1. Introduction

Cephalosporins are broad-spectrum β -lactam antibiotics, which are widely used in clinical treatment. However, they are prone to cause allergic reactions [1–3]. In particular, cephalosporins for injection have the highest incidence of allergic reactions [4]. A large number of clinical trials and researches have confirmed that poly-

merized impurities in β -lactam antibiotics can cause immediate hypersensitivity reaction, therefore, strictly controlling the polymerized impurities in cephalosporins is of great importance for clinical safety [5,6].

Reversed-phase liquid chromatography (RP-LC) is an efficient and highly specialized separation system [7]. However, preparation and standardization of polymerized impurities reference substance are very difficult [8]. And the polymerized impurities are strongly retained on the C18 column and difficult to elute. So it is hard to identify polymerized impurities from other impurities in the reversed-phase liquid chromatography [9]. Chinese

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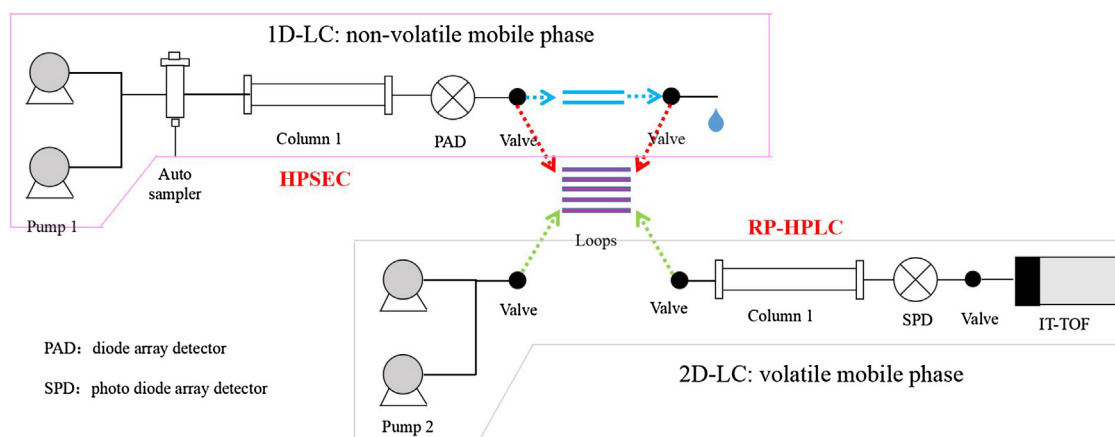


Fig. 1. A schematic of the instrumental set-up used for 2D HPSEC $\times$ LC-IT-TOF MS analyses, using two six-position, six-port valves equipped with six 20 μ L sample loops.

Pharmacopoeia (ChP 2015) has established a limited standard for polymerized impurities in cephalosporins, using size exclusion chromatography self-calibration method for the determination of the polymerized impurities in cephalosporins [10]. Currently the polymerized impurities are mainly determined by using a Sephadex G-10 gel filtration chromatography (GFC), however, this method presents low column efficiency, poor resolution and peak tailing [11]. On the contrary, high performance size exclusion chromatography (HPSEC) has the advantages of high separation efficiency, specificity, high sensitivity and short analysis time [12], which has a great advantage in the determination of polymerized impurity in cephalosporins.

The impurity of a drug is an important quality issue. For security reason, impurities that exceed 0.1% in a drug must be identified prior to clinical trial [13]. With the development of soft ionization techniques, electrospray ionization and multiple stage tandem mass spectrometry (ESI-MSⁿ) can provide considerable structural information by collision-induced dissociation reaction of precursor ions and has been more and more widely applied to analyze the structure of impurity [14]. Several LC methods have been reported for the determination of polymerized impurities in cephalosporins [3–5]. However, since the mobile phases contain non-volatile reagents, they are incompatible with MS.

The interest in two-dimensional liquid chromatography (2D-LC) has been developing rapidly since the last decades. In order to characterize the peaks obtained with the non-volatile system, after UV detector, fractions of each peak are trapped by a switching valve and transferred into a system with a volatile mobile phase before a MS detector [15]. Hao Luo et al. used 2D-LC as an on-line desalting tool allowing peptide identification directly from MS unfriendly HPLC methods [16]. This promising technique appears as a relevant solution for the contradiction between non-volatile mobile phase and mass spectrometry in pharmaceutical analysis. And Lulu Dai et al. effectively separated the impurity from the complex PEG oligomers and overcame the matrix interference, enabling mass spectrometric analysis of the impurity by a simple heart-cutting 2D-LC/MS approach [17]. Moreover, Jian Wang et al. had applied a trap-free two-dimensional liquid chromatography combined with ion trap/time-of-flight mass spectrometry to the separation and characterization of impurities and isomers in cefpiramide [18]. Trap-free two-dimensional liquid chromatography is more convenient, reproducible and rapid than other two-dimensional liquid chromatography because of the six quantitative loops and the absence of a trapping column. Besides, two-dimensional high performance size exclusion chromatography $\times$ reversed phase liquid chromatography coupled with ion trap time-of-flight mass spectrometry (2D HPSEC $\times$ LC-IT-TOF MS) effectively combined the

advantages of HPSEC and the ability of RP-HPLC to identify the polymerized impurities in cephalosporins. The schematic plot of the instrumental setup is shown in Fig. 1.

In this article, the structures of ten allergenic impurities in ceftazidime for injection, cefazolin sodium for injection, cefoperazone sodium and sulbactam sodium for injection and cefamandole nafate for injection were characterized by a trap-free 2D HPSEC $\times$ LC-IT-TOF MS with positive electrospray ionization mode. The structures and fragmentation pathways of ten allergenic impurities were investigated by complete fragmentation patterns. And the forming factors of polymerized impurity in cephalosporins were also studied. Determination of the polymerized impurities in cephalosporin antibiotics has been reported [3–5]. However, there is few articles concerning the structure identification of polymerized impurities in cephalosporins. 2D HPSEC $\times$ LC-IT-TOF MS method can be used to profile the polymerized impurities in cephalosporins, which is helpful to realize effective control over polymerized impurities in cephalosporins. Moreover, the peak sequence of impurities in the TIC chromatogram of LC-MS is identical with the LC chromatogram of the official analytical method due to the multiple heart-cutting 2D-LC approach and online desalting technique. Without changing the liquid phase condition (non-volatile mobile phase) of official analytical method, identification of impurity by 2D HPSEC $\times$ LC-IT-TOF MS can further improve the method of official monographs in pharmacopoeias and provide an evidence for pharmaceutical companies to declare drug. Hence present research work is undertaken considering general interest. We have done some research on the impurity spectrum and polymers of cephalosporins [13]. This article uses 2D HPSEC $\times$ LC-IT-TOF MS techniques to study the polymerized impurities of ceftazidime, cefazolin, cefoperazone and cefamandole. And the polymerized impurities obtained in this article have not been reported before.

2. Experimental

2.1. Materials

Ceftazidime for injection (batch number: 1702281) used in this study was provided by Hainan Hailing Pharmaceutical Co. Ltd. (Hainan, China). Cefazolin Sodium for injection (batch number: 10170501) used in this study was provided by Zhejiang Yatai Pharmaceutical Co. Ltd. (Shaoxing, China). Cefoperazone Sodium and Sulbactam Sodium for injection (batch number: A1608701-2) used in this study was provided by Harbin Pharmaceutical Group (Haerbin, China). Cefamandole Nafate for injection (batch number: 0211) used in this study was provided by Standard Pharmaceutical Co. Ltd. (Tainan, Taiwan). Acetonitrile was purchased from

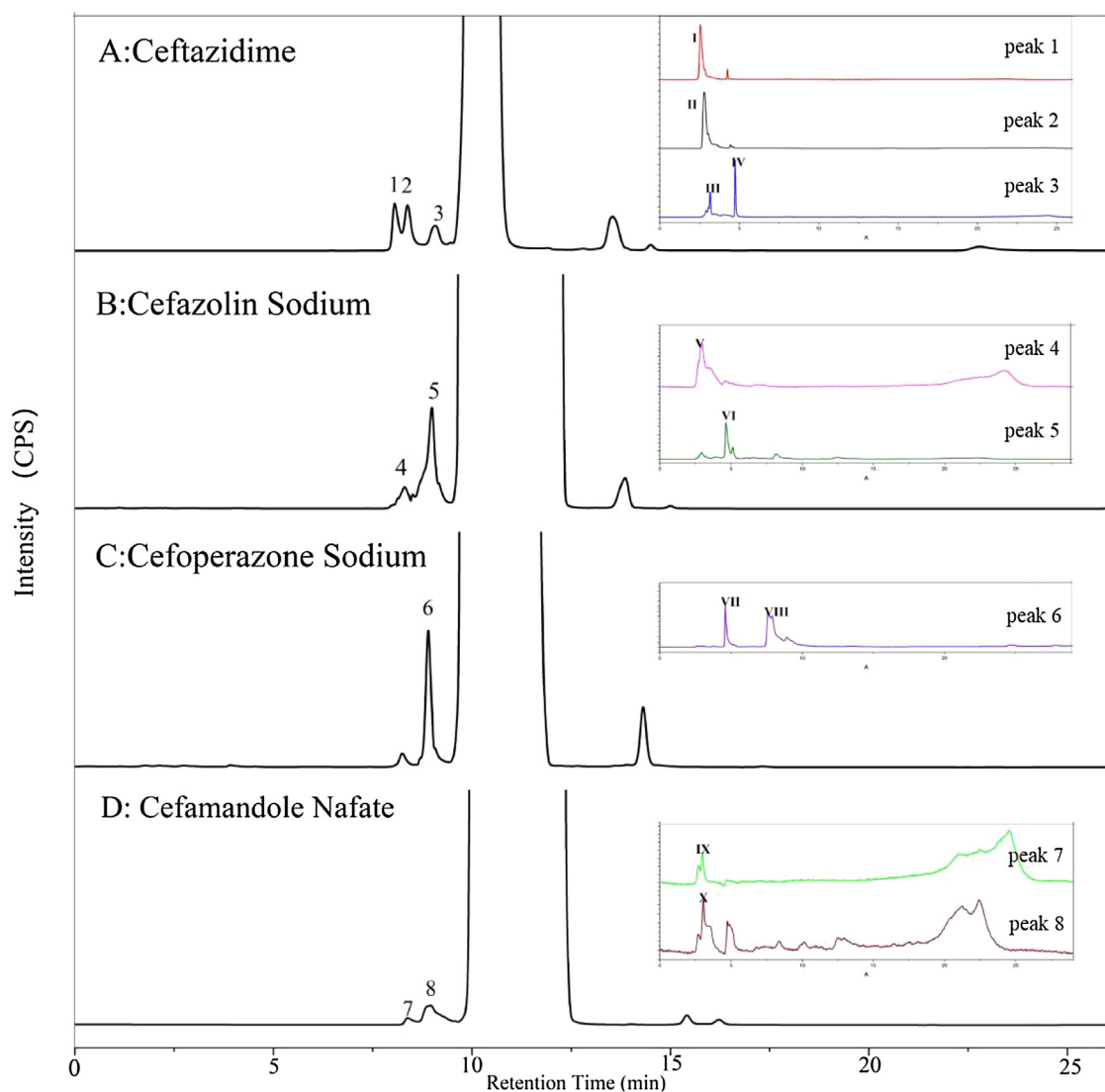


Fig. 2. First dimension high performance size exclusion chromatogram of ceftazidime for injection (A), cefazolin sodium for injection (B), cefoperazone sodium and sulbactam sodium for injection (C) and cefamandole nafate for injection (D). The insert pictures were the second dimension RPLC-UV chromatogram of eight polymer peaks. λ of detection = 254 nm.

Merck (Darmstadt, Germany), ammonium formate was purchased from Sigma-Aldrich (St. Louis, MO, USA), dibasic sodium phosphate (Analytical reagent) and sodium dihydrogen phosphate (Analytical reagent) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), and HPLC grade water was generated by a Millipore Milli-Q-Gradient purification system.

2.2. Instrument

2.2.1. Trap-free two-dimensional LC apparatus

A trap-free two-dimensional Nexera-XR liquid chromatograph (Shimadzu, Kyoto, Japan), a system equipped with four binary pumps was used. The first dimension included two binary pumps (LC-30AD), auto-sampler (SIL-30AC), column thermostat (CTO-20A) and diode array detector (SPD-M20A). Chromatographic separation in the first dimension 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 the first dimension: 0.005 mol L⁻¹ pH 7.0 buffer [0.005 mol L⁻¹ dibasic sodium phosphate solution – 0.005 mol L⁻¹ 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 1.00 mL min⁻¹ and the injection volume was 40 μ L. The second dimension was consisted of two binary pumps (LC-20AD), column thermostat (CTO-20A) and photo diode array detector (SPD-20A). Chromatographic separation in the second dimension was carried out at 40 °C 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 first and second dimension columns were connected by means of two high pressure six-position and six-port switching valves, and equipped with six 20 μ L stainless steel quantitative loops. The experimental configuration used for the 2D-LC is illustrated in Fig. 1. In the first dimension liquid chromatography, the analytes were trapped by the switching valves and stored in the six quantitative loops with the non-volatile mobile phase. Then, the trapped analytes in quantitative loops were transferred to the second analytical column with the volatile mobile phase in the second dimension liquid chromatography. The valve before the MS was used to control whether the substances were eluted to MS or to waste [19].

2.2.2. MS spectrometry

Trap-free two-dimensional liquid chromatography coupled to ion trap/time-of-flight mass spectrometer (2D HPSEC $\times$ LC-IT-TOF

Table 1

ESI–MS exact mass data and theoretical mass data on $[M+H]^+$ of ten impurities of ceftazidime for injection, cefazolin sodium for injection, cefoperazone sodium and sulbactam sodium for injection and cefamandole nafate for injection in the positive ion mode^a.

Imp.	formula	$[M+H]^+ (m/z)$	Theoretical $[M+H](m/z)$	Deviation (ppm)
I	C ₃₄ H ₃₆ N ₁₀ O ₁₅ S ₄	953.1270	953.1317	–4.93
II	C ₁₇ H ₁₉ N ₅ O ₈ S ₂	486.0721	486.0748	–5.55
III	C ₁₈ H ₁₆ N ₆ O ₅ S ₂	461.0715	461.0696	4.12
IV	C ₂₆ H ₂₈ N ₈ O ₉ S ₃	693.1192	693.1214	–3.17
V	C ₁₉ H ₂₂ N ₈ O ₈ S ₄	619.0508	619.0516	–1.29
VI	C ₁₁ H ₁₂ N ₆ O ₅ S	341.0669	341.0663	1.76
VII	C ₂₃ H ₂₅ N ₅ O ₉ S	548.1431	548.1446	–2.74
VIII	C ₂₃ H ₂₅ N ₅ O ₉ S	548.1425	548.1446	–3.83
IX	C ₁₈ H ₂₄ N ₄ O ₆ S ₄	521.0632	521.0651	–3.64
X	C ₁₆ H ₁₈ N ₂ O ₇ S	383.0909	383.0907	0.52

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

MS) was from Shimadzu Corp. (Kyoto, Japan). The mass spectrometry detector (MSD) was equipped with an electrospray ionization (ESI) source. The ionization mode was positive. 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 desolvation line (CDL) and heat block: 200 °C; detector voltage: 1.56 V; scan range: 100–2000 m/z ; the mode of MSⁿ: manual to MSⁿ.

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

The drugs of ceftazidime for injection, cefazolin sodium for injection, cefoperazone sodium and sulbactam sodium for injection and cefamandole nafate for injection were dissolved by water and the concentration was 10 mg mL^{–1}.

3. Results and discussion

3.1. Selection of chromatographic conditions

Based on the methods for the determination of polymerized impurity in the same kind of cephalosporins in 2015 edition of Chinese Pharmacopoeia [10], three phosphate buffer solutions at pH 7.0 with concentrations at 0.005 mol L^{–1}, 0.05 mol L^{–1} and

Table 2

ESI–MSⁿ exact mass data of major product ions of ten allergic impurities in positive ion mode.

Imp.	$[M+H]^+ (m/z)$	MS ² fragmentation ions (m/z)	MS ³ fragmentation ions (m/z)
I	953.1270	909.1200, 892.0933, 486.0659, 468.0551, 442.0751, 424.0671	
II	486.0721	468.0587, 442.0828, 424.0714, 398.0936, 323.0247	424.0752, 398.0945
III	461.0715	443.0613, 324.0063	280.0166, 262.9922, 427.0154
IV	693.1192	614.0570	
V	619.0508	406.9845, 341.0605	
VI	341.0669	322.0511, 298.0504	
VII	548.1431	406.0723	
VIII	548.1425	406.0718	
IX	521.0632	229.0393	
X	383.0909	339.1028, 321.0916, 292.0626	321.0912, 303.0827, 292.0656, 274.0564

0.1 mol L^{–1} were selected for test in this study. Fig. S1 showed the first dimension HPSEC–UV chromatograms of ceftazidime for injection, cefazolin sodium for injection, cefoperazone sodium and sulbactam sodium for injection, cefamandole nafate for injection with different concentrations of phosphate buffer solution. Compared with the chromatograms of different concentrations of phosphate buffer solution, the higher the ionic strength of the mobile phase, the later the retention time of the main peak. Under the premise of ensuring effective separation, the phosphate buffer system with lower ionic strength was selected as the mobile phase. A 0.005 mol L^{–1} pH 7.0 phosphate buffer solution was chosen as the mobile phase in the first dimension system which could achieve better separation and shorter retention time.

In order to permit the use of liquid chromatography–mass spectrometry analysis, salt used in the second dimension mobile phase must be a volatile salt. Ammonium formate was a volatile salt which could enhance the ionization capacity in both positive and negative ion modes. By testing, it was found that the retention time was appropriate and the phosphate was easily eluted when using (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) in second dimension [19].

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

Fig. 2 showed the high performance size exclusion chromatogram of ceftazidime for injection (A), cefazolin sodium for injection (B), cefoperazone sodium and sulbactam sodium for injection (C) and cefamandole nafate for injection (D) in the first dimension, eight polymer peaks (peaks 1–8) before the main peaks could be separated by the chromatographic condition in the first dimension system with the detection wavelength of 254 nm. Fig. S2 and the insert figure in Fig. S2 showed the first and second dimension chromatogram of blank control. In order to further check out the existence of polymers in peaks 1–8 and confirm their structures, the effluent of each peak (peaks 1–8) before the main peak on the high performance size exclusion chromatographic system was switched to the second dimension chromatographic system to obtain RPLC–UV chromatogram of eight polymer peaks (seen the insert figure in Fig. 2) and analyzed by LC–MS. There were ten impurities could be separated in the second dimension and their structures were deduced based on the high-resolution MSⁿ data, in which four impurities were polymerized impurities. Table 1 showed ten impurities of ceftazidime for injection, cefazolin sodium for injection, cefoperazone sodium and sulbactam sodium for injection and cefamandole nafate for injection in the positive ion mode. Table 2 showed ESI–MSⁿ exact mass data of major product ions of ten allergic impurities in the positive ion mode. The

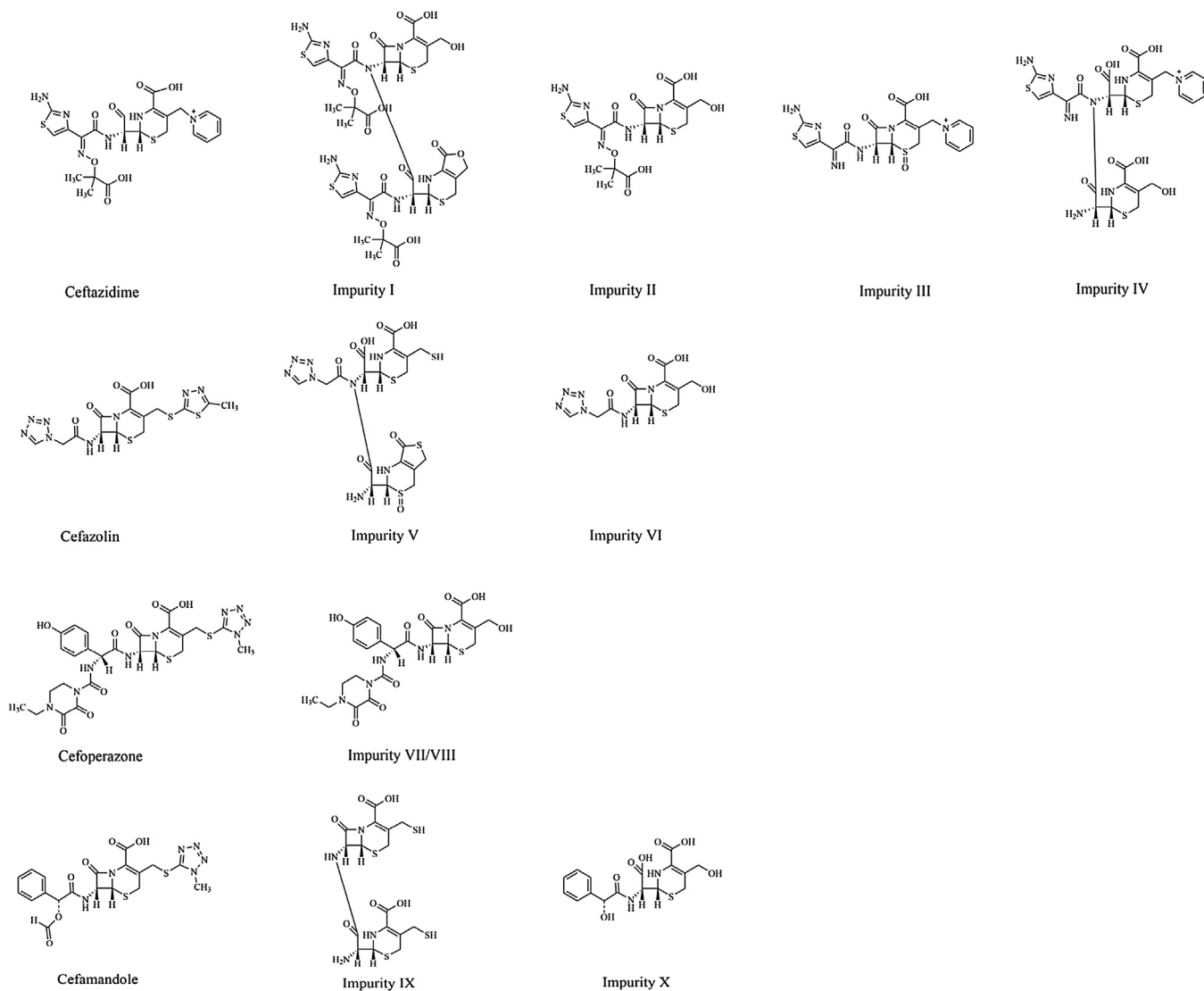


Fig. 3. Chemical structures of ceftazidime, cefazolin, cefoperazone, cefmenoxime and impurities I–X.

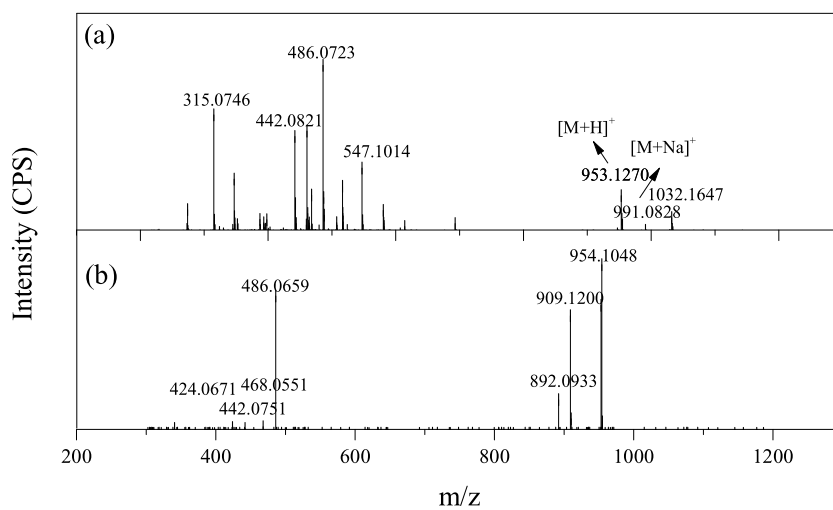


Fig. 4. (a) Full scan spectrum of impurity I showing $[M+H]^+$ ions at m/z 953.1270. (b) MS² spectrum of m/z 953.1270 ion.

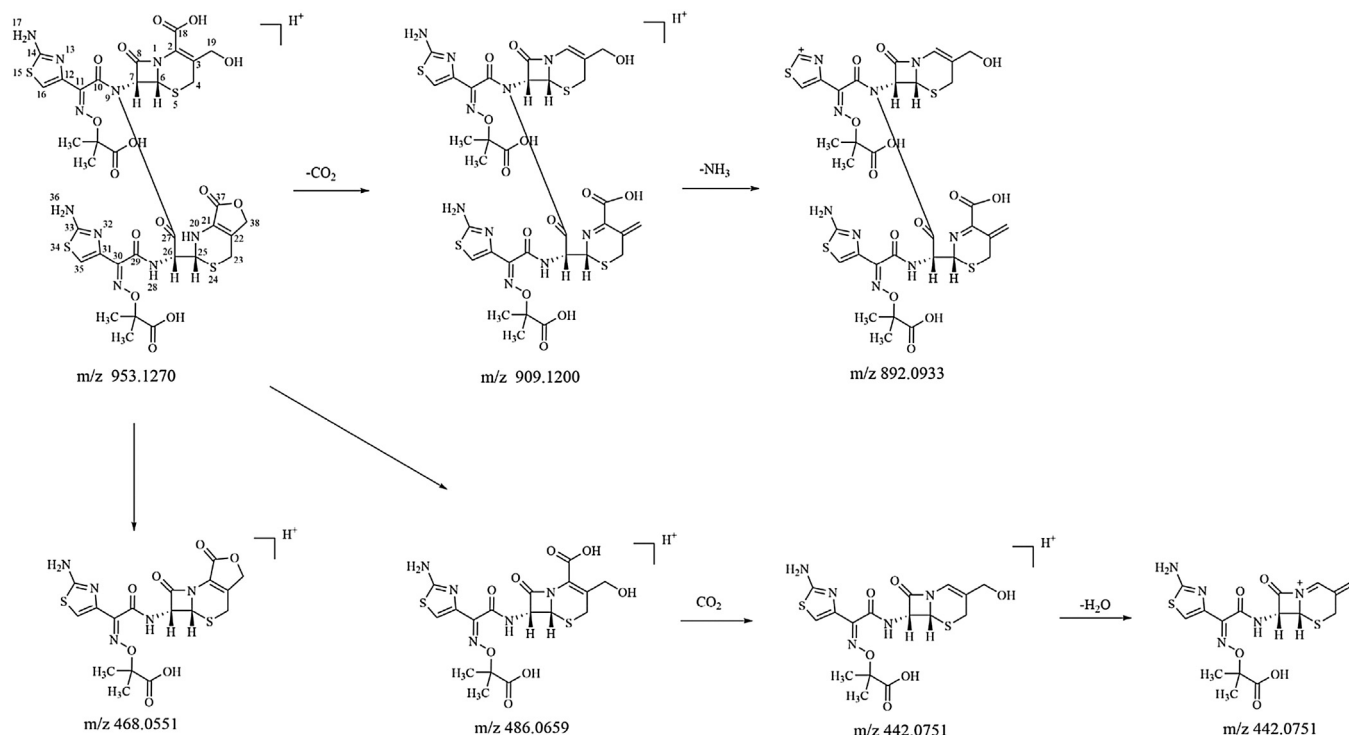


Fig. 5. Proposed fragmentation pathways of impurity I in the positive ion mode.

proposed chemical structures of ceftazidime, cefazolin, cefoperazone, cefmenoxime and ten allergenic impurities were shown in Fig. 3.

3.3. Structure elucidation

3.3.1. Ceftazidime for injection

3.3.1.1. Impurity I. The formula of impurity I was $\text{C}_{34}\text{H}_{36}\text{N}_{10}\text{O}_{15}\text{S}_4$ based on the data from TOF high resolution mass spectrometer. Fig. 4 revealed that $[\text{M}+\text{H}^+]$ at m/z 953.1270 and $[\text{M}+\text{Na}^+]$ at m/z 991.0828 appeared in the full scan of ESI^+ . The plausible fragmentation pathway of impurity I in the positive ion mode was illustrated in Fig. 5. The protonated molecule at m/z 953.1270 of impurity I fragmented into the product ion at m/z 909.1200 by means of the loss of 44 Da, corresponding to CO_2 , which could further lose one NH_3 (17 Da) to form the fragment ion at m/z 892.0933. Another diagnostic fragmentation pathway of protonated molecule at m/z 953.1270 concerned the amide bond cleavage between N-9 and C-27, leading to the ions at m/z 486.0659 and m/z 468.0551. From above information, it was concluded that impurity I was resulting from intermolecular aminolysis of β -lactam ring by the amine of the first molecule, and one molecule in the structure of impurity I was that the pyridine at the position of C-19 in the structure of ceftazidime was substituted by hydroxyl group, and the other one was that the pyridine at the C-19 position of ceftazidime was lost accompanied by forming of the ester with a carboxyl group at the C-2 position [20].

3.3.1.2. Impurity II. The formula of impurity II was $\text{C}_{17}\text{H}_{19}\text{N}_5\text{O}_8\text{S}_2$ based on the data from TOF high resolution mass spectrometer. The plausible fragmentation pathway of impurity II in the positive ion mode was illustrated in Fig. S3. The fragmentation pathway of the ions at m/z 442.0828 and m/z 424.0714 was the same as that of the ion at m/z 486.0659 in impurity I. The fragment ions at m/z 398.0936 and m/z 323.0247, which were produced by the

loss of CO_2 , $\text{C}_3\text{H}_6\text{O}$ (58 Da) and NH_3 from the ion at m/z 442.0828, confirmed a non-modified at the side chain of C-7 for impurity II. From the above information, it was concluded that impurity II was a degradation product of ceftazidime by the hydroxyl group substituting pyridine at the position of C-19.

3.3.1.3. Impurity III. The formula of impurity III was $\text{C}_{18}\text{H}_{16}\text{N}_6\text{O}_5\text{S}_2$ based on the data from TOF high resolution mass spectrometer. The plausible fragmentation pathway of impurity III in the positive ion mode was illustrated in Fig. S4. Impurity III showed a protonated molecule ion at m/z 461.0715. In the collision induced dissociation (CID) spectrum, the ion at m/z 443.0613 fragmented into the characteristic ions at m/z 324.0063 and m/z 280.0166 by the loss of one pyridine, oxygen and CO_2 , respectively, supporting that an oxygen addition occurred on the sulfur atom of the hydrogenated thiazide ring. From the above information, it was concluded that the oxygen addition occurred on the sulfur of the hydrogenated thiazide ring of ceftazidime with the hydrolysis of hydroxylamine bond at the side chain of C-11. And impurity III was deduced as an oxidative degradation product of ceftazidime.

3.3.1.4. Impurity IV. The formula of impurity IV was $\text{C}_{26}\text{H}_{28}\text{N}_8\text{O}_9\text{S}_3$ based on the data from TOF high resolution mass spectrometer. The plausible fragmentation pathway of impurity IV in the positive ion mode was illustrated in Fig. S5. The protonated molecule ion at m/z 693.1192 fragmented into the ion at m/z 614.0570 by the neutral loss of one pyridine and forming a five-membered ring, with consecutive bond cleavage between C-6 and C-7 and neutral loss of one sulfur and one $\text{C}_6\text{H}_5\text{NO}_2\text{S}$ structure to form the fragment ion at m/z 427.0154. Based on the information above, the structure of impurity IV was resulting from intermolecular aminolysis of β -lactam ring by the amine of the first molecule. One molecule in the structure of impurity IV was formed by the hydrolysis of amide bond in β -lactam ring and hydroxylamine bond at the side chain of C-11 in the structure of ceftazidime, and the other one was

formed by the hydrolysis of amide bond at the side chain of C-7 in the structure of ceftazidime accompanied by the hydroxyl group substituting pyridine at the side chain of C-3.

3.3.2. Cefazolin sodium for injection

3.3.2.1. Impurity V. The formula of impurity V was $C_{19}H_{22}N_8O_8S_4$ based on the data from TOF high resolution mass spectrometer. The plausible fragmentation pathway of impurity V in the positive ion mode was illustrated in Fig. S6. The protonated molecule at m/z 619.0508 initially gave rise to the ion at m/z 341.0605 due to the cleavage of amide bond and loss of one H_2S . Another fragmentation pathway of the ion at m/z 619.0508 concerned the loss of 213 Da, corresponding to $C_3H_2N_4O$, H_2S , H_2O , sulfur and oxygen, conducting to the ion at m/z 406.9845. Based on the information above, the structure of impurity V was resulting from intermolecular aminolysis of β -lactam ring by the amine of the first molecule. One molecule was formed by the hydrolysis of thioether bond in the structure of cefazolin, the other one was formed by the hydrolysis of thioether bond and amide bond at the side chain of C-7 in the structure of cefazolin accompanied by the oxygen addition occurred on the sulphurs.

3.3.2.2. Impurity VI. The full scan mass spectrum of impurity VI showed $[M+H]^+$ at m/z 341.0669 and $[M+K]^+$ at m/z 379.0237 in the positive ion mode, indicating that the molecular weight was 340. The elemental composition of impurity VI by exact mass measurements was confirmed as $C_{11}H_{12}N_6O_5S$. The plausible fragmentation pathway of impurity VI in the positive ion mode was illustrated in Fig. S7. In the CID spectrum of the ion at m/z 341.0669, it could lose two NH_3 (34 Da) to form the fragment ion at m/z 298.0504. Another fragmentation pathway of the protonated molecule at m/z 341.0669 concerned the loss of one H_2O and form a five-membered ring, leading to the ion at m/z 322.0511. From the above information, the structure of impurity VI was that the 2-mercapto-5-methyl-1,3,4-thiadiazole in the structure of cefazolin was replaced by hydroxyl group.

3.3.3. Cefoperazone sodium and sulbactam sodium for injection

3.3.3.1. Impurity VII and impurity VIII. TOF high resolution mass showed that impurity VII and impurity VIII had the same molecule species $[M+H]^+$ and same MS fragmentation. So they were suggested as isomers. The formula of impurity VII and impurity VIII were both $C_{23}H_{25}N_5O_9S$ based on the data from TOF high resolution mass spectrometer. The plausible fragmentation pathway of impurity VII/VIII in the positive ion mode was illustrated in Fig. S8. The product ion at m/z 406.0723 was probably formed by the cleavage of C–N bond at the side chain of C-11 and neutral loss of one $C_6H_{10}N_2O_2$ structure from the protonated molecule at m/z 548.1431. From the above information, the structures of impurity VII and impurity VIII were that the 5-mercapto-1-methyltetrazole in the structure of cefoperazone was replaced by hydroxyl group.

3.3.4. Cefamandole nafate for injection

3.3.4.1. Impurity IX. The formula of impurity IX was $C_{18}H_{24}N_4O_6S_4$ based on the data from TOF high resolution mass spectrometer. The plausible fragmentation pathway of impurity IX in the positive ion mode was illustrated in Fig. S9. The fragmentation pathway of the protonated molecule at m/z 521.0632 fragmented into the product ion at m/z 229.0393 by the cleavage of amide bond and neutral loss of one $C_9H_{12}N_2O_3S_2$ structure and one sulfur. These experiments revealed that the structure of impurity IX was resulting from intermolecular aminolysis of β -lactam ring by the amine of the first molecule. The molecules in the structure of impurity IX was formed by the hydrolysis of thioether bond at the side chain of C-3 and amide bond at the side chain of C-7 in the structure of cefamandole nafate.

3.3.4.2. Impurity X. The formula of impurity X was $C_{16}H_{18}N_2O_7S$ based on the data from TOF high resolution mass spectrometer. The plausible fragmentation pathway of impurity X in the positive ion mode was illustrated in Fig. S10. The fragment ion at m/z 321.0912 could be formed by neutral loss of one CO_2 (44 Da) and one H_2O (18 Da) from the protonated molecule at m/z 383.0909. Further fragmented into the diagnostic ion at m/z 303.0827 by the loss of one H_2O , confirming the hydrolysis of β -lactam ring for impurity X. From the above information, the structure of impurity X was that the 5-mercapto-1-methyltetrazole in the structure of cefamandole nafate was replaced by hydroxyl group accompanied by the hydrolysis of β -lactam ring.

4. Forming factors of polymerized impurity in cephalosporins

Cephalosporins were the most widely used in the family of antibiotics. Researches had shown that polymerized impurities could cause an immediate hypersensitivity reaction. Therefore, the study of the production factors of polymerized impurities in cephalosporins had become an important approach to reduce the reaction [2–5]. There are two ways for cephalosporins to produce polymerized impurity: 1) The polymerization reaction between the side chain and the mother nucleus: the polymerized impurity was resulting from intermolecular aminolysis of β -lactam ring by the amine of the second molecule. This was main polymerization reaction for the production of polymerized impurities in cephalosporins, and its reaction rate was affected by the tension energy and conjugate energy of the β -lactam ring [19]. By changing the opening rate of the β -lactam ring, it ultimately promoted or slowed down the formation of polymerized impurities. 2) The polymerization reaction between the side chains: the amino groups on the 7-side chain nucleophilic attacked the carbonyl on the side chain of another molecule to form polymerized impurity, such as the polymerized impurity in cefodizime [19].

The forming factors of polymerized impurity in cephalosporins were as follows: a): Increasing the effect of absorbing electrons of 3-side chain in the structure of cephalosporins enhanced the polymerization and led to easy formation of polymerized impurities, such as impurity I. Compared with cefazolin, cefoperazone and cefamandole, ceftazidime was more easily to produce polymerized impurity because of pyridine at the side chain of C-3 in the structure of ceftazidime having a strong electron-absorption. b): The steric hindrance of the 7-side chain contributed to prevent the formation of the polymerization, such as cefoperazone [21]. Because the larger substituents on the C-7 side chain resulted in an increase in steric hindrance, there was no polymerized impurity in cefoperazone sodium and sulbactam sodium for injection through the examination by 2D HPSEC $\times$ LC-IT-TOF MS. c): When there was no 3-side chain in cephalosporins or the 3-side chain was an alkyl group, it was easy to form a polymerized impurity with a molecular weight of 2 M ("M" meant the molecule weight of cephalosporin), such as cefadroxil [22]. d): When there was an ether bond at the 3-side chain, ether bond could be easily hydrolyzed or formed a five-membered ring with the carboxyl group at the position of C-2. Therefore, it was easy to form a polymerized impurity with a molecular weight of $[2M-R-H]$ or $[2M-R+OH]$, such as impurity I in ceftazidime. e): Since the polymerization reaction between the side chains need to lose a molecule of water, the molecular weight of the obtained polymerized impurity was 18 Da less than that of the polymerized impurity obtained by the polymerization reaction between the side chain and the mother nucleus.

5. Conclusions

Ten allergenic impurities in ceftazidime for injection, cefazolin sodium for injection, cefoperazone sodium and sulbactam sodium for injection and cefamandole nafate for injection were separated and characterized by a trap-free 2D HPSEC $\times$ LC-IT-TOF MS with positive mode of electrospray ionization method for the improvement of official method. Moreover, the 2D HPSEC $\times$ LC-IT-TOF MS presented the following advantages: 1. By the utilization of the multiple heart-cutting 2D-LC approach and online desalting technique, the contradiction between non-volatile mobile phase and mass spectrometry was completely solved. 2. The effectiveness of the conventional HPSEC method was identified by 2D HPSEC $\times$ LC-IT-TOF MS and the structures of polymerized impurities found by HPSEC were characterized by LC-IT-TOF MS. Besides, The main cause of the polymerized impurity in cephalosporins were also elucidated. The polymerization reaction between the side chain and the mother nucleus was the main formation of polymerized impurity in cephalosporins, which was susceptible to the electron-absorbing property of 3-side chain and the steric hindrance of the 7-side chain.

Acknowledgment

This research is supported by the food and drug science and technology project of Zhejiang food and drug administration in 2016 (No.YP201601).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jpba.2018.03.043>.

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Supporting information

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Figure caption:

Figure S1 First dimension high performance size exclusion chromatography of ceftazidime for injection, cefazolin sodium for injection, cefoperazone sodium and sulbactam sodium for injection and cefamandole nafate for injection with different concentrations of phosphate buffer solution, a- 0.005 mol·L⁻¹ for ceftazidime , b- 0.05 mol·L⁻¹ for ceftazidime, c- 0.1 mol·L⁻¹ for ceftazidime; d- 0.005 mol·L⁻¹ for cefazolin; e- 0.05 mol·L⁻¹ for cefazolin; f- 0.1 mol·L⁻¹ for cefazolin; g- 0.005 mol·L⁻¹ for cefoperazone; h- 0.05 mol·L⁻¹ for cefoperazone; i- 0.1 mol·L⁻¹ for cefoperazone; j- 0.005 mol·L⁻¹ for cefamandole; k- 0.05 mol·L⁻¹ for cefamandole; l- 0.1 mol·L⁻¹ for cefamandole. λ of detection = 254 nm.

Figure S2 The first dimensional chromatogram of blank control, The insert figure was the second dimensional chromatogram of blank control.

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 positive 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 positive ion mode.

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

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

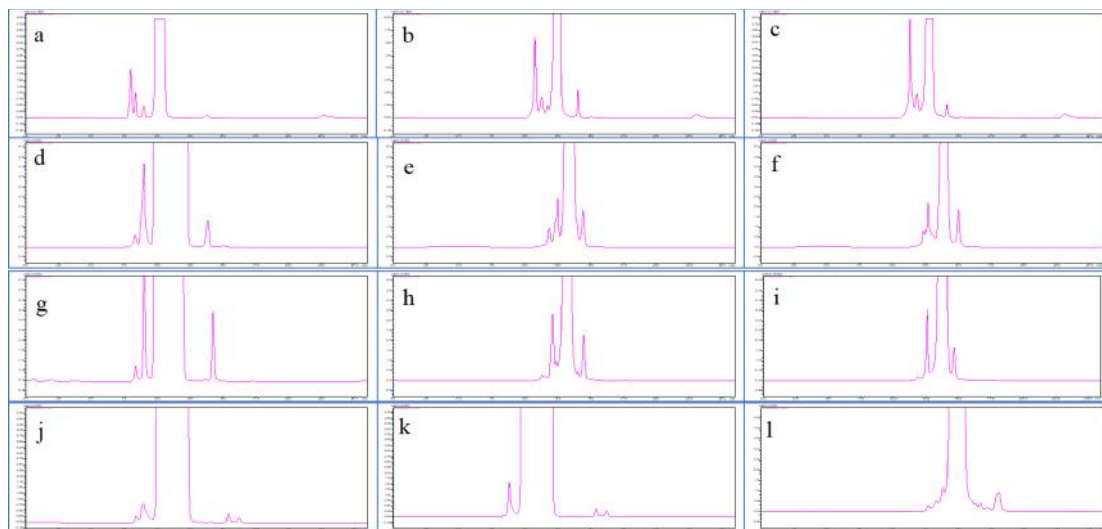


Figure S1 First dimension high performance size exclusion chromatography of ceftazidime for injection, cefazolin sodium for injection, cefoperazone sodium and sulbactam sodium for injection and cefamandole nafate for injection with different concentrations of phosphate buffer solution, a- $0.005 \text{ mol} \cdot \text{L}^{-1}$ for ceftazidime , b- $0.05 \text{ mol} \cdot \text{L}^{-1}$ for ceftazidime, c- $0.1 \text{ mol} \cdot \text{L}^{-1}$ for ceftazidime; d- $0.005 \text{ mol} \cdot \text{L}^{-1}$ for cefazolin; e- $0.05 \text{ mol} \cdot \text{L}^{-1}$ for cefazolin; f- $0.1 \text{ mol} \cdot \text{L}^{-1}$ for cefazolin; g- $0.005 \text{ mol} \cdot \text{L}^{-1}$ for cefoperazone; h- $0.05 \text{ mol} \cdot \text{L}^{-1}$ for cefoperazone; i- $0.1 \text{ mol} \cdot \text{L}^{-1}$ for cefoperazone; j- $0.005 \text{ mol} \cdot \text{L}^{-1}$ for cefamandole; k- $0.05 \text{ mol} \cdot \text{L}^{-1}$ for cefamandole; l- $0.1 \text{ mol} \cdot \text{L}^{-1}$ for cefamandole. λ of detection = 254 nm.

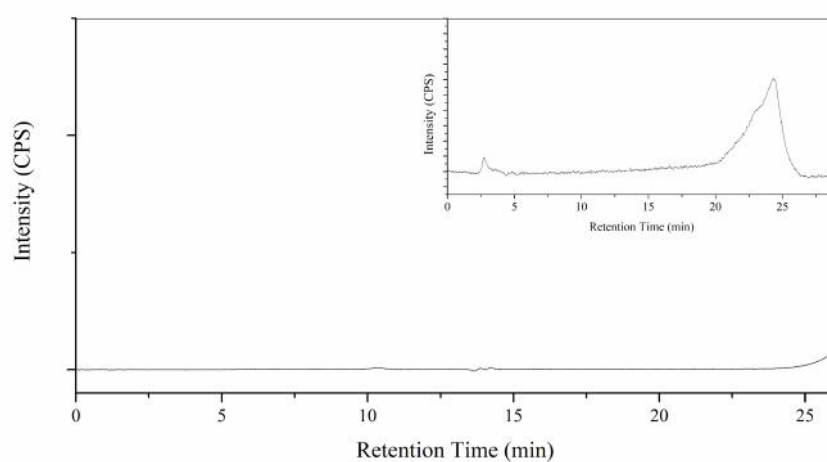


Figure S2 The **first dimension chromatogram** of blank control, The insert figure was the second dimensional chromatogram of blank control.

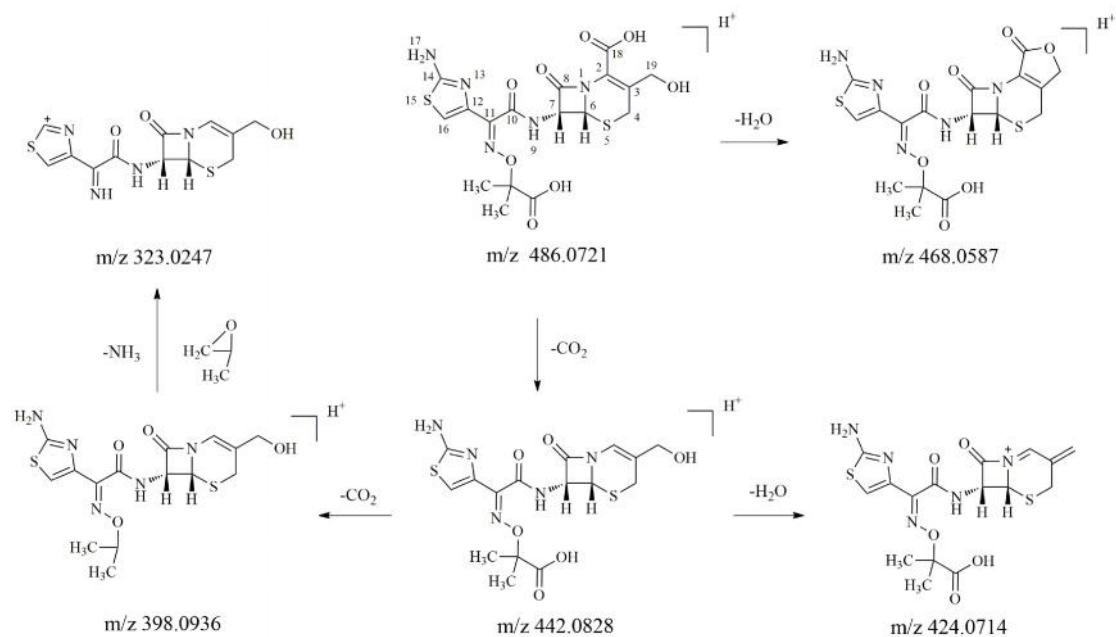


Figure S3 proposed fragmentation pathways of impurity in the positive ion mode.

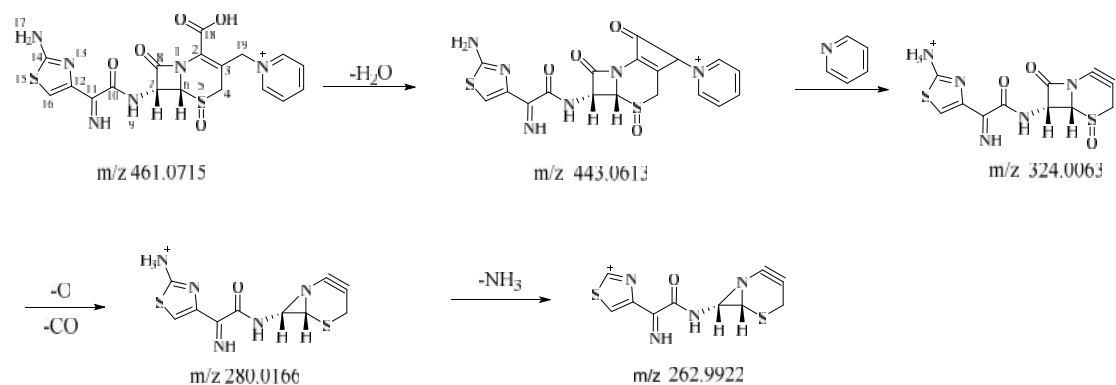


Figure S4 proposed fragmentation pathways of impurity in the positive ion mode.

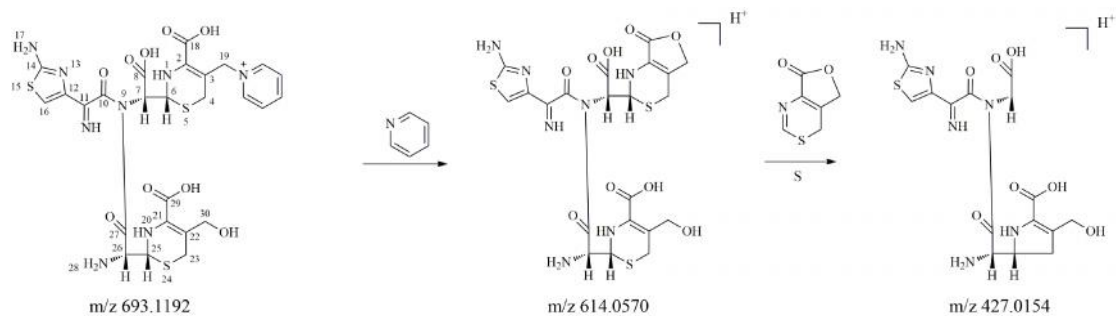


Figure S5 proposed fragmentation pathways of impurity in the positive ion mode.

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

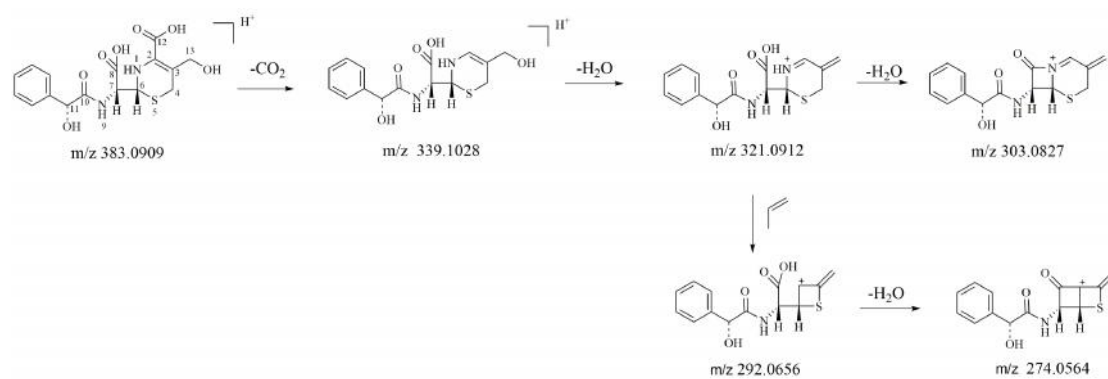


Figure S10 proposed fragmentation pathways of impurity in the positive ion mode.