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Analysis of Polymer Impurities in Cephalosporin Antibiotics

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Abstract [**Objectives**] To establish a HPLC-MS method for the determination of polymer impurities in cefathiamidine and its preparations. [**Methods**] Kromasil 100-5 C_{18} column (4.6 mm × 250 mm, 5 μm) was used for analysis; mobile phase ammonium acetate solution (pH 6.30)-acetonitrile, gradient elution; volumetric flow rate 1.0 mL/min; column temperature 40 ℃; multi-reaction monitoring mode was used for analysis, and positive ion scanning was chosen as the electrospray ion source. [**Results**] The resolution between impurities and main peaks under this method was greater than 1.5, and 8 known impurities and 2 polymer impurities could be completely separated and distinguished. It was inferred that the molecular ion peak $[M+H]^+$: *m/z* 727.187 4, *m/z* 785.193 7 was the possible polymer impurity of this product. [**Conclusions**] A method for the analysis of polymer impurities in cefathiamidine and its preparations was formed, which could achieve the purpose of simultaneous analysis of small molecule impurities and polymer impurities, and could better control the content of single impurities in the polymer, providing a reliable inspection basis for strict control of cefathiamidine quality.

Key words Cefathiamidine, Cephalosporin polymer impurities, Impurity analysis, High Performance Liquid Chromatography-Mass Spectrometry

1 Introduction

Cefathiamidine is the first generation cephalosporin, and it has a strong effect on Gram-positive bacteria. Cefathiamidine can also form high molecular polymers, especially with strong antibacterial activity against Staphylococcus and Enterococcus, and is widely used in clinical application[1]. Its basic structure is the same as many semi-synthetic β-lactam antibiotics on the market, and it will cause immediate allergic reaction in clinical use, which is extremely harmful to patients[2]. The occurrence of allergic reaction can be controlled by controlling polymer impurities[3]. The existing national drug standards for cefathiamidine for injection do not list cefathiamidine polymer as a verification item, and domestic pharmaceutical research mainly focuses on the synthesis and purification of cefathiamidine[4-5], but there are few studies on the determination of purity, content and related impurities of cefathiamidine and its preparations, as well as the detection of its degradation products[6]. In addition to polymer impurities, cefathiamidine also has some small molecule impurities[7]. From the perspective of clinical drug safety, China has required the detection of polymers in the quality inspection of such drugs, and the detection, analysis and control of impurities in drugs are the focus of drug quality control[8].

Some studies have used molecular exclusion chromatography to detect cefathiamidine impurities, which can detect polymer impurities. However, in the actual application process, it is found that due to the many types of impurities in cefathiamidine, the resolution of some impurities is poor, and there are multiple small molecule impurities in one impurity peak at the same time, so the specificity of this method is poor[3]. In addition, HPLC method[9] is used to separate and determine the polymer impurities in cefathiamidine and its preparations, in which the polymer impurities are dimer, trimer and polymer formed by cefathiamidine, 7-ACA, cefathiamidine β-lactam ring hydrolysate and cefathiamidine β-lactam ring hydrolysate decarboxylate. This method uses spherical hydrophilic silica gel as filler and buffer salt-organic phase solution as mobile phase. This method has the advantages of good resolution, simplicity, rapidity, strong specificity and high sensitivity, but this method only limits the total quantity of impurities whose retention time is less than that of cefathiamidine peak, and does not consider the influence of small molecule impurities on detection, which is not conducive to controlling the content of individual impurities in the polymer.

This research topic provides a method for the detection of polymer impurities in cefathiamidine and its preparations, which can separate and detect all potential impurities of cefathiamidine, realize the purpose of simultaneous analysis of small molecule impurities and polymer impurities, and better control the content of individual impurities in polymer.

2 Materials and methods

2.1 Instruments Liquid chromatography system (Agilent 1260); Q-TOF mass spectrometer (Agilent 6530).

2.2 Reagents Cefathiamidine (batch No.: LMA1911003, Baiyunshan Chemical Pharmaceutical Factory, Guangzhou Baiyunshan Pharmaceutical Group Co., Ltd.); cefathiamidine for injection (batch No.: SSJ200401, SSJ200404, Guangxi Kelun Pharmaceutical Co., Ltd.); reference preparation cefathiamidine for injection (batch No.: 190901, Guangzhou Baiyunshan Tianxin Pharmaceutical Co., Ltd.); cefathiamidine impurity A (batch No.: PITBLM-A-CP-20180528-01), cefathiamidine impurity B (batch No.: PITBLM-3-CP-20180601-01), cefathiamidine impurity D (batch No.: PITBLM-D-CP-20181206-01), oxidation impurity (batch No.: PITBLM-O-20180601-01), 6R, 7S-isomer (batch

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No.: PITBLM-7S-20180412-03) (Guangzhou Paipai Biotechnology Co., Ltd.); cefathiamidine impurity C (batch No.: 130669-201501) and 7-aminocephalosporanic acid (batch No.: 130538-200902) (China Institute of Food and Drug Control); 1,3-Diisopropylthiourea (batch No.: VOTLH, TCL); citric acid (AR analytically pure); acetonitrile (HPLC chromatographic grade).

2.3 Solution preparation

2.3.1 Test solution. 10 mg of cefathiamidine test sample was accurately weighed and the cefathiamidine test article solution diluted to 0.5 mg/mL was dissolved with water.

2.3.2 Standard solution (impurity location solution). 2.5 mg of cefathiamidine impurities A, B, C, D, oxidation impurities, 6R, 7S-isomer, 7-aminocephalosporanic acid, and 1,3-diisopropylthiourea reference samples were taken separately, mixed with diluent, diluted to volume, and shaken well to obtain the reference mother solution; 0.5 mL of the mother solution of impurity C and 1 mL of the mother solution of other impurities except impurity C were respectively pipetted into 10-mL volumetric flask to obtain the control solution (impurity location solution). The diluent was a mixed solution of phosphate buffer and acetonitrile (90 : 10). The phosphate buffer was prepared as follows: 2.76 g of anhydrous disodium hydrogen phosphate and 1.29 g of citric acid were dissolved in water and diluted to 1 000 mL.

2.3.3 System suitability solution. 10 mg of cefathiamidine raw material was accurately weighed, and 2, 1, and 2 mL of mother solution of impurities A, C, and D were added respectively; diluent was added to dissolve and it was quantitatively diluted to produce a mixed solution containing 12.5 μg of impurity C and 25 μg of impurities A and D per 1 mL, as a system suitability solution.

2.4 Detection and analysis of polymer impurities in cefathiamidine and its preparations by HPLC-MS

2.4.1 HPLC conditions. Kromasil 100-5 C_{18} column (4.6 mm × 250 mm, 5 μm), mobile phase A: 0.01 mol/L ammonium acetate solution, glacial acetic acid was added to adjust pH to 6.30, mobile phase B: acetonitrile, gradient elution, flow rate 1.0 mL/min, detection wavelength 254 nm, column temperature 40 ℃, injection volume 20 μL, elution procedure is shown in Table 1, and three chromatographic conditions were used for detection.

Table 1 HPLC gradient elution procedure

Chromatographic condition 1			Chromatographic condition 2			Chromatographic condition 3		
Time//min	Mobile phase A//%	Mobile phase B//%	Time//min	Mobile phase A//%	Mobile phase B//%	Time//min	Mobile phase A//%	Mobile phase B//%
0	90	10	0	90	10	0	90	10
15	90	10	15	90	10	15	90	10
35	70	30	39	84	16	39	84	16
40	70	30	53	60	40	53	60	40
55	20	80	68	20	80	68	20	80
65	20	80	78	20	80	98	20	80
70	90	10	83	90	10	103	90	10
75	90	10	90	90	10	110	90	10

2.4.2 Mass spectrometry conditions. Ion source type: electrospray ion source (Dual AJS ESI), positive ion mode scanning; temperature of atomizing gas: 320 ℃; drying gas flow rate 8 L/min; atomizing gas pressure: 35 psi; sheath gas temperature: 350 ℃; sheath gas flow rate: 11 L/min; capillary end voltage: 120 V; ion scanning range: MS: 100 – 1 100 *m/z*; MS^2: 100 – 1 100 *m/z*; bombarding voltage: 10, 20, 30V; sampling frequency: MS: 2 Spectra/s; MS^2: 3 Spectra/s.

2.5 Determination of cefathiamidine impurities and polymer impurities in its preparations by molecular exclusion chromatography Spherical hydrophilic silica gel was used as filler (TSK-GEL G2000SWXL, 7.8 mm × 30 cm, 5 μm); the mobile phase was 0.01 mol/L ammonium acetate solution-acetonitrile (93 : 7), the flow rate was 0.5 mL/min, and the detection wavelength was 254 nm. 20 μL of impurity location solution and 20 μL of system suitability solution were injected into the liquid chromatograph respectively.

3 Results and analysis

3.1 Optimization results of HPLC conditions The chromatogram obtained by testing the test solution and blank solvent (water) under the three chromatographic conditions is shown in Fig. 1. It can be seen from the figure that impurity D was included in the main peak of the test solution in the method of chromatographic condition 1; under chromatographic condition 2, the resolution between impurity D and main peak was greater than 1.5; after extending the elution time of high proportion organic phase on the basis of chromatographic condition 2, that is, chromatographic condition 3, the impurity spectrum of the test product was consistent with that of chromatographic condition 2; this indicated that after the high proportion of organic phase was extended for a long time, no new impurities were eluted, and chromatographic condition 2 had sufficient elution ability, so chromatographic condition 2 was finally selected.

3.2 Results of isolation and localization of cefathiamidine impurity by HPLC-MS The impurity location solution, system suitability solution and blank solvent (water) were taken for injection and testing analysis, and the results are shown in Fig. 2. As can be seen from Fig. 2, this detection method can completely separate and distinguish 8 known impurities.

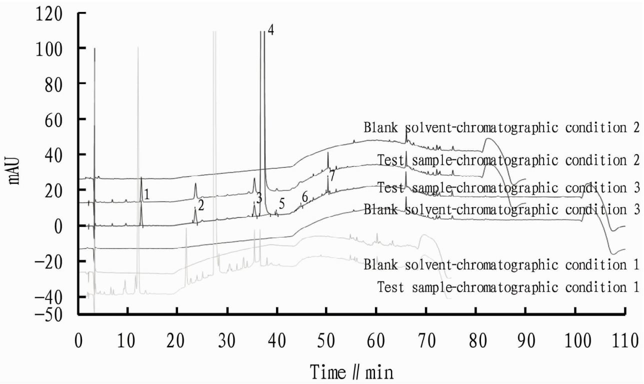

Fig. 1 Chromatogram of test sample under different chromatographic conditions

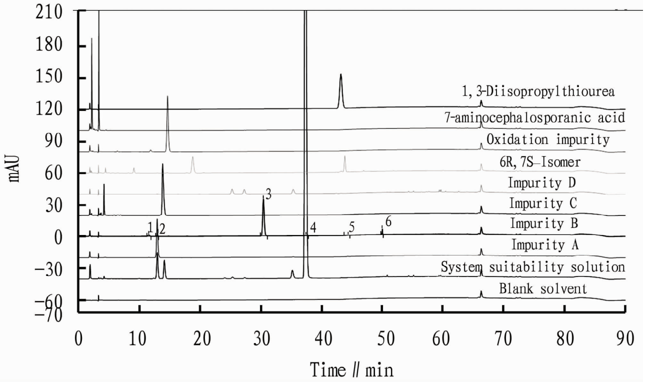

Fig. 2 Chromatogram of 8 known impurities

3.3 Results of separation of polymer impurities in cefathiamidine and its preparation by molecular exclusion chromatography

The test results are shown in Fig. 3. From the results, it can be seen that impurity C and 7-aminocephalosporanic acid both peaked before the main peak, and the resolution with the main peak was good; 4 impurities (oxidation impurity, impurity A, impurity B and 6R,7S-cefathiamidine isomer) all peaked at the main peak position, and the resolution was poor; 1,3-diisopropylthiourea and impurity D peaked after the main peak, and the resolution from the main peak was good; most impurities peaked at the main peak position, with poor resolution, and multiple small molecule impurities existed at the same time in one impurity peak, with poor specificity.

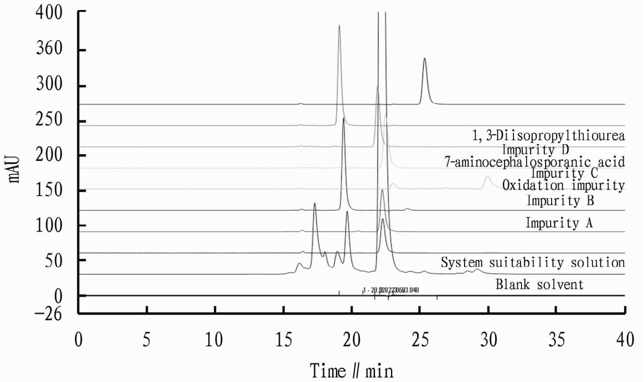

Fig. 3 Location chromatogram of each known impurity detected by molecular exclusion chromatography

3.4 Content calculation results by area normalization method

The self-made preparation of cefathiamidine for injection, the reference preparation and the raw material of cefathiamidine were tested, and the content was calculated by the area normalization method. The test results are shown in Table 2. Known impurity A, impurity D and 1,3-diisopropylthiourea were detected in self-made preparation of cefathiamidine for injection, the reference preparation and the raw material of cefathiamidine, and some unknown impurities were also detected.

Table 2 Results of content detected by area normalization method

Peak	Self-made preparation test results			Reference preparation Test results			Raw material of cefathiamidine test results		
	RT // min	RRT	Content // %	RT // min	RRT	Content // %	RT // min	RRT	Content // %
Unknown impurity 1	24.010	0.64	0.13	11.630	0.31	0.03	9.440	0.26	0.03
Impurity A	12.937	0.35	0.14	12.927	0.35	0.08	12.400	0.34	0.25
Unknown impurity 2	40.213	1.08	0.02	24.007	0.64	0.06	22.957	0.63	0.14
Unknown impurity 3	44.897	1.21	0.03	25.277	0.68	0.03	33.947	0.94	0.01
Impurity D	35.180	0.94	0.14	35.183	0.94	0.29	34.657	0.96	0.05
Main peak	37.243	1.00	N/A	37.240	1.00	N/A	36.187	1.00	N/A
Unknown impurity 4	50.233	1.35	0.03	29.963	0.80	0.03	39.157	1.08	0.01
1,3-Diisopropylthiourea	43.437	1.17	0.01	43.283	1.16	0.02	43.017	1.19	0.01
Unknown impurity 5	–	–	–	48.947	1.31	0.03	49.783	1.38	0.03

3.5 HPLC-MS analysis of cefathiamidine test samples

3.5.1 MS test results of cefathiamidine. The Q-TOF data analysis results are shown in Table 3, where *m/z* 473.152 5 was the $[M+H]^+$ ion peak of cefathiamidine, and its molecular formula was determined by high-resolution mass spectrometry to be $C_{19}H_{29}N_4O_6S_2^+$, and the unsaturation degree was 7. In summary, the inferred structure of cefathiamidine $[M+H]^+$ and its $[M+H]^+$ ion MS^2 fragmentation pathway are shown in Fig. 4. The observed mass number of each ion in the MS spectra was consistent with the theoretical exact mass number of each structural ion shown in Fig. 4 (with a deviation of less than 10 ppm).

Table 3 Analysis results of high-resolution mass spectrometry data of cefathiamidine

Molecular formula	Diff (abs. ppm)	Experimental mass	Theoretical mass	Number of nitrogens	DBE
$C_{19}H_{29}N_4O_6S_2^+$	0.4	473.152 5	473.152 3	Even	13
$C_{17}H_{25}N_4O_4S_2^+$	1.2	413.131 7	413.131 2	–	–
$C_{15}H_{25}N_4OS_2^+$	0.9	341.146 7	341.146 4	–	–
$C_8H_9N_4O_3S^+$	1.0	201.105 8	201.105 6	–	–
$C_7H_{15}N_2^+$	2.4	127.123 3	127.123 0	–	–

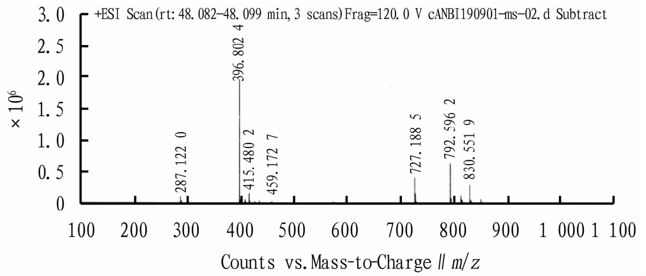

Fig. 4 Structure of cefathiamidine [M + H]$^+$ and mass spectrum of its MS^2 fragmentation pathway

3.5.2 MS and MS^2 test results for unknown impurity *m/z* 727.187 4. The full scan spectrum of *m/z* 727.187 4 and the MS^2 fragmentation spectrum (secondary mass spectrum) of [M + H]$^+$ ion are shown in Fig. 5-6. The full scan results showed that *m/z* 727.187 4 was [M + H]$^+$ ion of unknown impurity, its molecular formula was $C_{29}H_{39}N_6O_{10}S_3^+$, and its unsaturation was 13. Like cefathiamidine, the C-C bond of the unknown impurity was broken, losing the *m/z* 623.177 5 fragment ion formed by the substituent acetic acid at position 3, and *m/z* 595.182 6 was an ion fragment formed by the opening of the *m/z* 623.177 5 four-membered ring and the removal of one-molecule methyl group; the daughter ion *m/z* 201.105 6 was the same as the daughter ion of cefathiamidine, indicating that the unknown impurity also contained the same structure of this part.

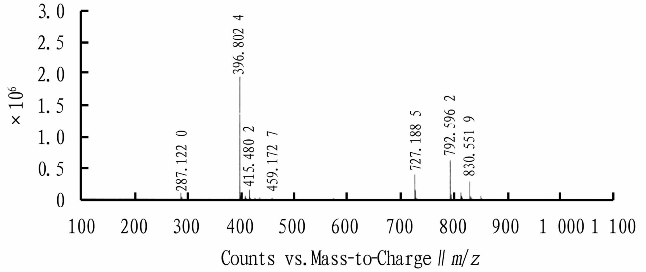

Fig. 5 Full scan mass spectrum of *m/z* 727.187 4

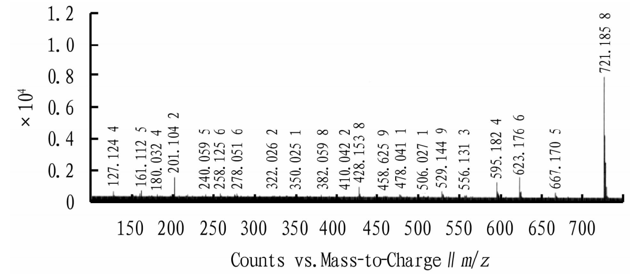

Fig. 6 Secondary mass spectrum of *m/z* 727.187 4

In summary, the inferred structure of *m/z* 727.187 4 unknown impurity and its [M + H]$^+$ ion MS^2 fragmentation pathway are shown in Fig. 7. The observed mass number of each ion in the MS spectra was consistent with the theoretical exact mass number of each structural ion shown in Fig. 7 (with a deviation of less than 10 ppm).

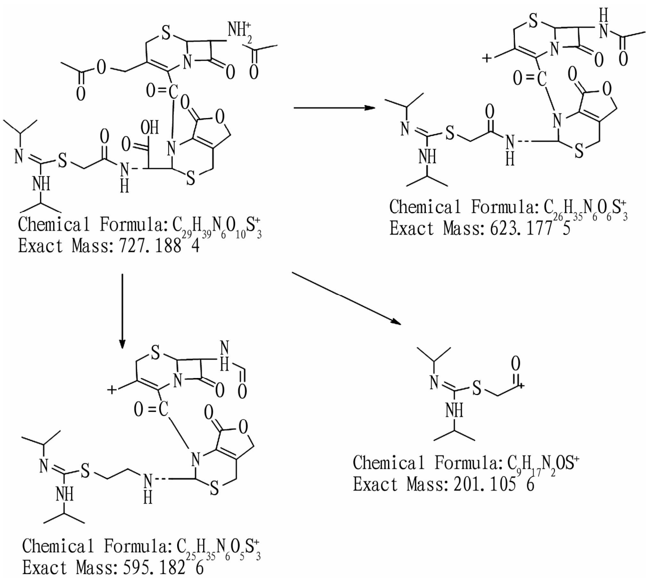

Fig. 7 Fragmentation pathway of compound with [M + H]$^+$ at *m/z* 727.187 4 in MS^2

3.5.3 MS and MS^2 test results for unknown impurity *m/z* 785.193 7. The full scan spectrum of *m/z* 785.193 7 and the MS^2 fragmentation spectrum (secondary mass spectrum) of [M + H]$^+$ ion are shown in Fig. 8-9. The full scan results showed that *m/z* 785.193 7 was [M + H]$^+$ ion of unknown impurity, its molecular formula was $C_{31}H_{41}N_6O_{12}S_3^+$, and its unsaturation was 15. Like cefathiamidine, the unknown impurity C-C bond was broken, losing the *m/z* 681.183 0 fragment ion formed by the substituent acetic acid at position 3, or by removing one-molecule carboxylic acid from the four-membered ring, and *m/z* 653.188 0 was the ionic fragment formed by removing one-molecule CO from *m/z* 681.183 0; the daughter ion *m/z* 201.105 6 and *m/z* 127.123 0 were the same as the daughter ion of cefathiamidine, indicating that the unknown impurity also contained the same structure of this part.

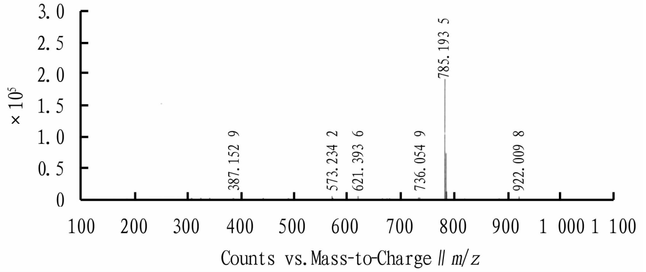

Fig. 8 Full scan mass spectrum of *m/z* 785.193 7

In summary, the inferred structure of *m/z* 785.193 7 unknown impurity and its [M + H]$^+$ ion MS^2 fragmentation pathway are shown in Fig. 10. The observed mass number of each ion in the

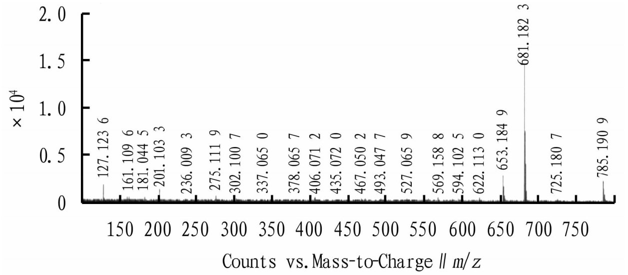

Fig. 9 Secondary mass spectrum of *m/z* 785.193 7

MS spectra was consistent with the theoretical exact mass number of each structural ion shown in Fig. 10 (with a deviation of less than 10 ppm).

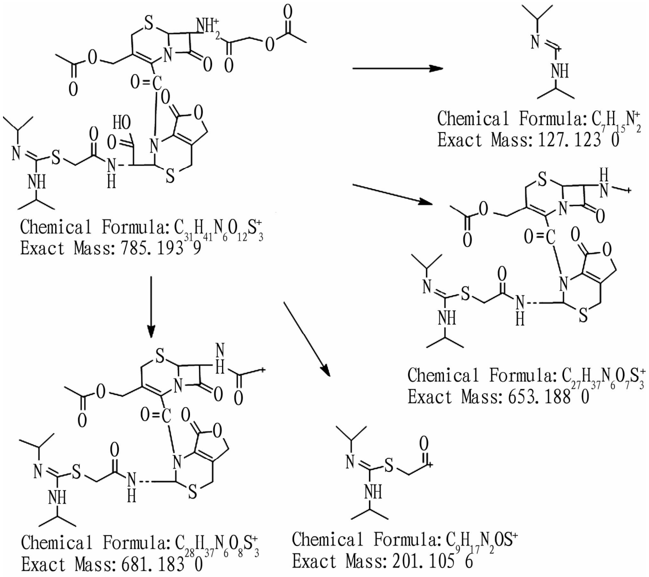

Fig. 10 Fragmentation pathway of compound with $[M+H]^+$ at *m/z* 785.193 7 in MS^2

4 Conclusions and discussion

The specificity of molecular exclusion chromatography for the analysis of polymer impurities in cefathiamidine is poor, and it is impossible to comprehensively separate and accurately characterize polymer and small molecule impurities[10-13]. Studies[3] have shown that using phosphate buffer-acetonitrile as the mobile phase, among the four main chromatographic peaks before the retention time of the main peak of cefathiamidine, three chromatographic peaks include both small molecule compounds and a variety of non-polar macromolecule compounds, which have not been effectively separated in their chromatographic system. In this study, the chromatographic conditions for the detection of polymer impurities in cefathiamidine and its preparations were optimized, and 0.01% ammonium acetate-acetonitrile was used as the mobile phase. The results showed that adding appropriate amount of acetic acid to the mobile phase was helpful in ionizing cefathiamidine and improving the peak shape. Cefathiamidine highly responded, the peak shape was sharp and symmetrical, and the repeatability was good. At the same time, gradient elution was selected to ensure that the peak shape of the target substance was not broadened, and there was no leading peak or tailing peak. The best gradient elution procedure for the detection of polymer impurities in cefathiamidine and its preparations by high performance liquid chromatography-mass spectrometry was chromatographic condition 2 (Section **3.1** for details). Under this chromatographic condition, the resolution of impurity D and the main peak was greater than 1.5, and it had sufficient elution ability to better control the content of individual impurities in the polymer, which provided a reliable test basis for strictly controlling the quality of cefathiamidine. In this study, the area normalization method was used to calculate the content, and all known impurity A, impurity D and 1,3-diisopropylthiourea were detected, and some unknown impurities were also detected. Compared with the external standard method[11], this method had good repeatability and reproducibility and good advantages of simple operation and no impurity reference substance was needed. The purpose of simultaneously separating, detecting and qualitatively analyzing all potential small and macromolecular impurities of cefathiamidine in the same chromatographic system was achieved, and the structure of polymer impurities could be obtained by inference, including 8 known impurities of cefathiamidine and 2 polymer impurities. According to the results of the study, it was inferred that *m/z* 727.187 4, *m/z* 785.193 7, the molecular ion peak $[M+H]^+$ in raw material, self-made preparation and reference preparation, were possible polymer impurities in this product. The molecular formula of the compound with *m/z* 727.187 4 was $C_{29}H_{39}N_6O_{10}S_3^+$, with unsaturation of 13; the molecular formula of the compound with *m/z* 785.193 7 was $C_{31}H_{41}N_6O_{12}S_3^+$, with unsaturation of 15.

By studying the structure of the polymer impurities of cefathiamidine, the structure of the polymer impurities of cefathiamidine was analyzed based on the structural properties of cefathiamidine, the mass spectrum information of the impurities and the polymerization reaction mechanism of cefathiamidine can be preliminarily speculated, providing a reference for subsequent research on cefathiamidine polymers and the formation mechanism of polymer impurities in antibiotics, to better control the content of individual impurities in the polymer. At the same time, it has important reference significance for the impurity spectrum study and quality control of cefathiamidine raw materials and preparations, and is helpful in comparing the quality differences of related substances and polymers of cephalosporin antibiotics from different manufacturers, as well as the advantages and disadvantages of production process control.

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fathiamidine. This is of great significance to ensure the safety and effectiveness of drugs and the safety of patients, and provides a strong guarantee for the quality control of Cefathiamidine raw materials and their preparations.

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