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

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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 × 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 × 30 cm, 5 μm), 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 × 150 mm, 3.5 μm) 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 MS^n 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.

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].

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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 20ul 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 doxycyline 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 × 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 μl 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 × 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 × 30 cm, 5 μm). 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 μL. 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 × 2.1 mm, 1.9 μm). 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 × 150 mm, 3.5 μm). 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 × 150 mm, 3.5 μm). 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 μL 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 μL 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