Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 937–944

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

The stability of biapenem and structural identification of impurities in aqueous solution

Ming Xia[a,*], Tai-Jun Hang[b], Fei Zhang[a], Xiao-Min Li[a], Xiang-Yang Xu[a]

[a] Department of Pharmacy, Jiangsu Simcere Pharmaceutical R&D Co., Ltd., Xuanwu Avenue No. 699-18, Nanjing 210042, PR China
[b] Department of Pharmaceutical Analysis, China Pharmaceutical University, Nanjing 210009, PR China

ARTICLE INFO

Article history:
Received 23 November 2008
Received in revised form 4 February 2009
Accepted 4 February 2009
Available online 13 February 2009

Keywords:
Biapenem
Stability
Degradation products
Dimeric impurity
HPLC–MS

ABSTRACT

The stability of biapenem in aqueous solution was investigated. Forced degradation of biapenem was carried out under different concentrations, pH values and temperatures. The degradation products were determined by reverse-phase HPLC and identified by LC–MS/MS. One dimeric impurity was obtained by reverse-phase preparative HPLC and characterized by LC–MS/MS and NMR. A possible degradation mechanism has been presented.

1. Introduction

Biapenem is a novel synthetic 1β-methyl carbapenem antibiotic that shows a broad spectrum antibacterial activity against Gram-positive and Gram-negative aerobic and anaerobic bacteria. Biapenem is generally well tolerated and in comparison with imipenem, meropenem and panipenem, it is more stable to human renal dihydropeptidase-I (DHP-I) [1].

It is of common knowledge that the impurities of β-lactam antibiotics are important with respect to the quality of the products. Smith et al. studied the stability and kinetics of degradation of imipenem in aqueous solution, characterizing the structure of impurities with nuclear magnetic resonance (NMR) spectroscopy [2]. In addition, gel filtration chromatography (GFC) has been reported for the separation of polymerized impurities in meropenem [3]. Cai et al. developed an optimized routine quality control method for separation and analysis of amoxicillin high molecular weight impurities by means of different gel media for GFC [4]. Ranadive et al. also investigated the formation, isolation and identification of oligomers of aztreonam with GFC [5]. Furthermore, a liquid chromatography–tandem mass spectrometric (LC–MS/MS) method has been developed for the identification of dimeric impurities in ampicillin and amoxicillin [6]. Dimeric degradation products in concentrated ertapenem solution were isolated by preparative high-performance liquid chromatography (HPLC) [7]. High-performance capillary electrophoresis (HPCE) has also been successfully applied to monitor the degradation of carbapenems (imipenem, panipenem and meropenem) [8].

However, to our best knowledge the stability of biapenem and the identification of degradants in aqueous solution have not yet been reported. Our present work has focused on this.

2. Experimental

2.1. Materials

Biapenem samples were supplied by Nanjing Simcere Dongyuan Pharmaceutical Co., Ltd. (Jiangsu, China). Ammonium acetate and acetonitrile were HPLC grade, obtained from Kermel Chemical Reagent Co., Ltd. (Tianjin, China) and Tedia Company Inc. (Fairfield, OH, USA), respectively. Other reagents such as acetic acid and aqueous ammonia were AR grade (Kermel, Tianjin, China). Water used was Milli-Q grade (Millipore). D_2O and sodium tetramethylsilylpropanoate (TSP) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

2.2. High-performance liquid chromatography

An Agilent 1200 Series HPLC system equipped with a diode array detector and Chemstation data handling system (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for analysis. The analytical

* Corresponding author. Tel.: +86 25 85566666x1777; fax: +86 25 855666666x1771.
E-mail addresses: xiaming1217@163.com, xiaming1217@yahoo.com.cn (M. Xia).

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doi:10.1016/j.jpba.2009.02.002

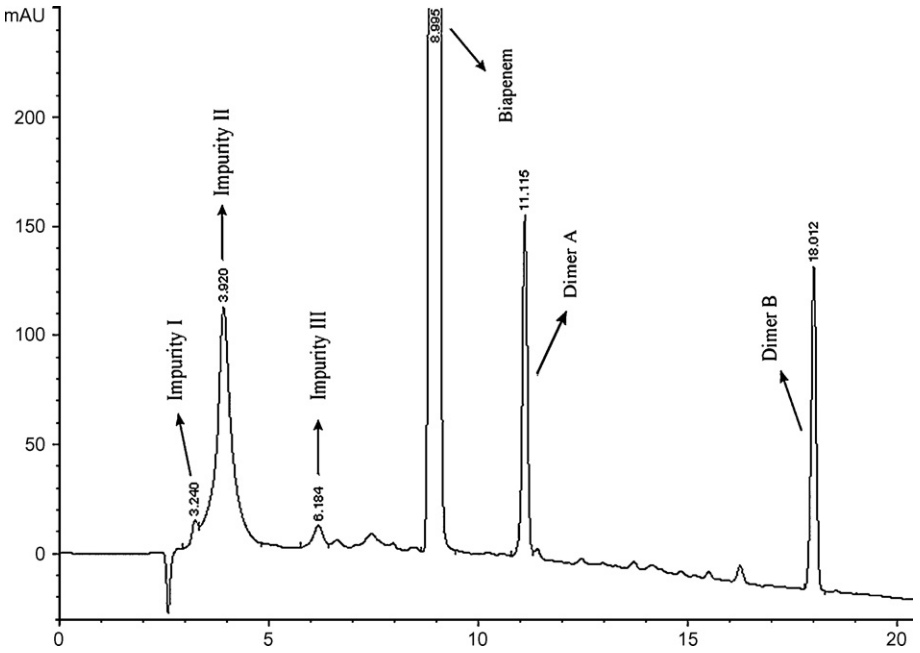

Fig. 1. Typical HPLC profile of biapenem solution with impurities. Concentration = 20 mg/ml, heated at 80 °C for 5 min. For HPLC conditions see Section 2.2.

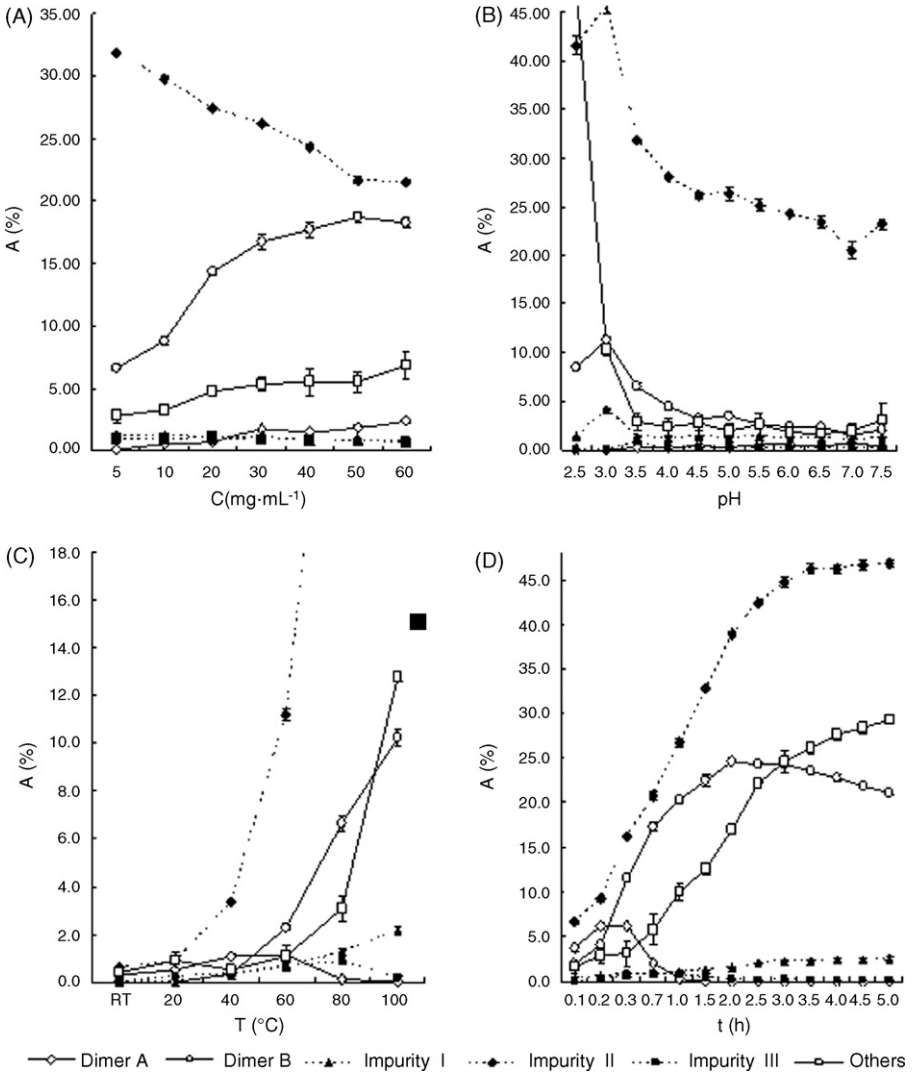

Fig. 2. Influencing factors on biapenem degradation in aqueous solution. (A) Concentration: 40 min, 80 °C, pH 3.5. (B) pH: 40 min, 80 °C, 5 mg/ml. (C) Temperature: 40 min, 5 mg/ml, pH 3.5. (D) Time: 50 mg/ml, 80 °C, pH 3.5. Each point and vertical bar represents the mean ± standard error for each impurity's area percentage in HPLC.

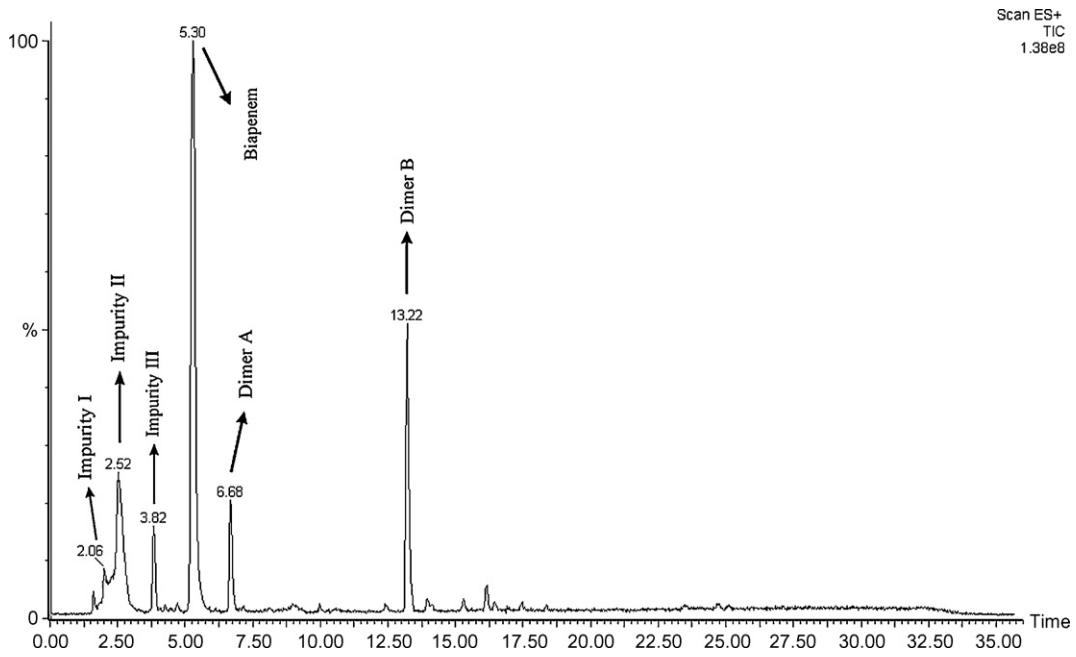

Fig. 3. Total ion chromatogram of biapenem aqueous solution. Concentration = 10 mg/ml, heated at 80 °C in water for 5 min. For HPLC conditions see Section 2.2.

column was Waters Sunfire C_{18} (4.6 mm × 150 mm, 5 μm) column. Mobile phase A was 0.01 M ammonium acetate. Mobile phase B was acetonitrile. The flow rate was kept at 1.0 ml/min and the column was maintained at room temperature. The injection volume was 20 μl. Related substances were detected at 220 nm using a gradient HPLC method. The A:B (v/v) linear gradient elution program was as follows: 99:1 (0 min); 70:30 (27 min); 70:30 (30 min); 99:1 (31 min); and 99:1 (36 min).

2.3. LC–MS/MS analysis

LC–MS/MS analysis was performed using Waters Quattro micro API triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA), operating under positive electrospray ionization (ESI) mode. MassLynx V4.0 was used for data acquisition and data processing. The HPLC conditions were the same as in Section 2.2.

The typical source conditions were: capillary voltage, 3.5 kV; cone voltage, 40 V; source temperature, 150 °C; desolvation temperature, 380 °C desolvation gas flow, 550 l/h; cone gas flow 50 l/h. High pure nitrogen was used as nebulizer gas while helium as auxiliary gas. The collision energies were 15–25 eV.

2.4. Preparative high-performance liquid chromatography

A Waters 600 Semi-Preparative Liquid Chromatographic system equipped with 2487 UV–VIS detector (Waters Corporation, Milford, MA, USA) was used for the isolation of dimers. Chromatographic data was analyzed by Empower pro data handling system. A Waters Sunfire C_{18} (10 mm × 150 mm, 10 μm) preparative column was used. Mobile phase consisted of 0.01 M ammonium acetate and acetonitrile in the ratio of 93:7. The flow rate was 5 ml/min and the injection volume was 0.7 ml. The detection was carried out at 220 nm.

2.5. Isolation and preparation of Dimer B

An aqueous solution of biapenem (20 mg/ml, adjusted to pH 3.5 with acetic acid) was degraded at 80 °C for 1.5 h. Dimer B was isolated by semi-preparative HPLC, using the conditions described in Section 2.4.

The fractions were collected at regular intervals of time and each fraction was analyzed by HPLC under the conditions described in Section 2.2. The fractions of >98% were pooled together and concentrated under high vacuum at ambient temperature using a rotary evaporator. The amount of acetonitrile in the fractions was removed. The fractions were frozen at −20 °C for 12 h. A Lyo-0.4 Freeze Dryer (Tofflon Science and Technology Co., Ltd., Shanghai, China) was used to lyophilize the frozen fractions at a controlled temperature of −50 °C for 12 h. Dimer B was obtained as yellow powder with chromatographic purity >97%. The isolated Dimer B was subjected to analysis by ESI–MS/MS and NMR.

2.6. NMR spectroscopy

The ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) spectra were recorded on Bruker AVANCE AV-500 spectrometer (Bruker Group, Fällanden, Switzerland) using D_2O as solvent and TSP as internal standard.

3. Results and discussion

3.1. HPLC analyses

A typical HPLC profile of biapenem aqueous solution is shown in Fig. 1. Impurity I, Impurity II and Impurity III were eluted before biapenem. Impurity I and Impurity II could not be separated indicating that they might be isomers. Dimer A and Dimer B were well separated, following the peak of biapenem.

3.2. Forced degradations of biapenem in aqueous solution

For quality control purposes, the stability of carbapenems in aqueous solution has been widely studied. It had been previously reported that the degradation of imipenem and meropenem fol-

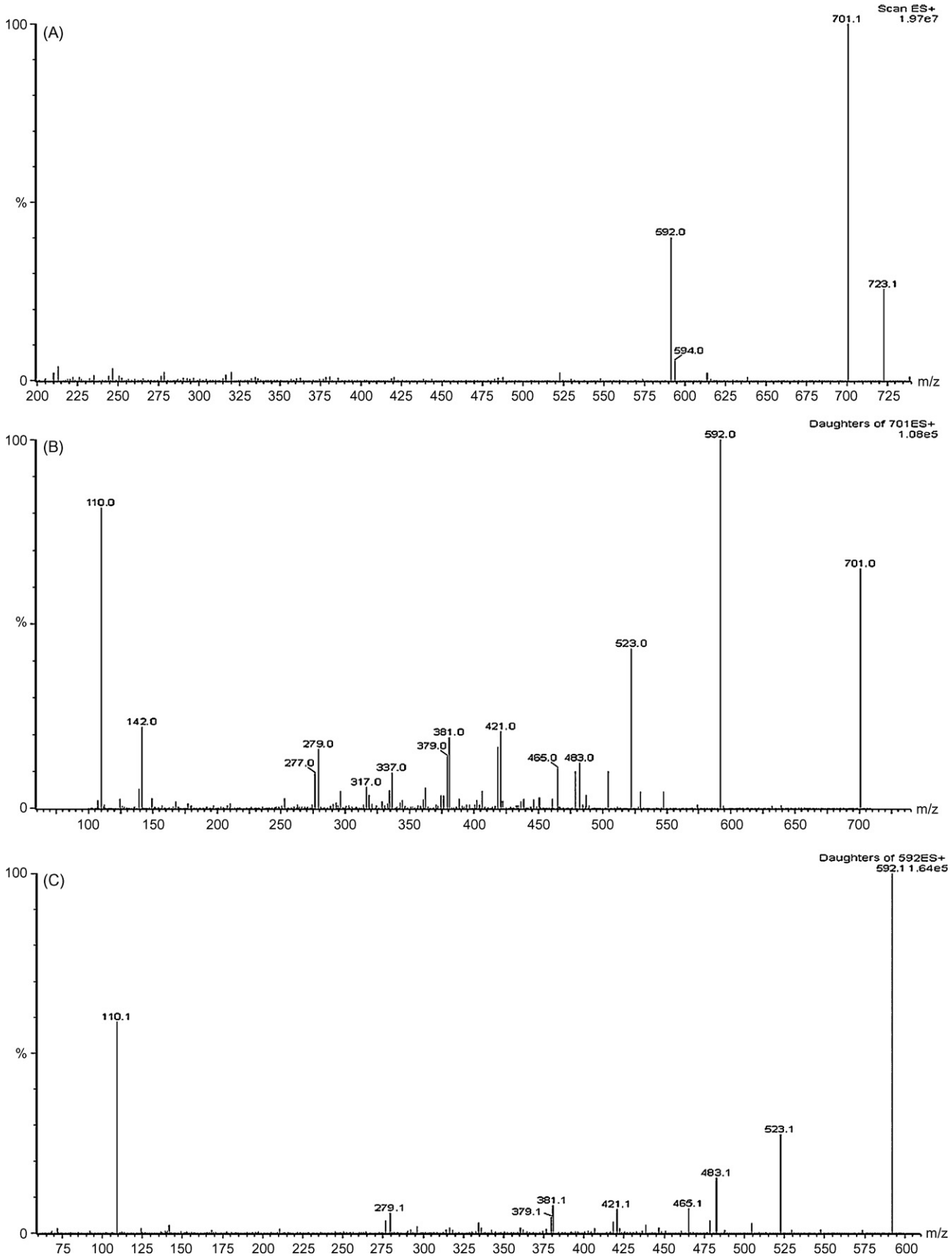

Fig. 4. LC–MS/MS analysis of Dimer B. (A) MS full scan of Dimer B. (B) MS^2 on m/z 701 ion at normalized collision energy of 25 eV. (C) MS^2 on m/z 592 ion at normalized collision energy of 20 eV.

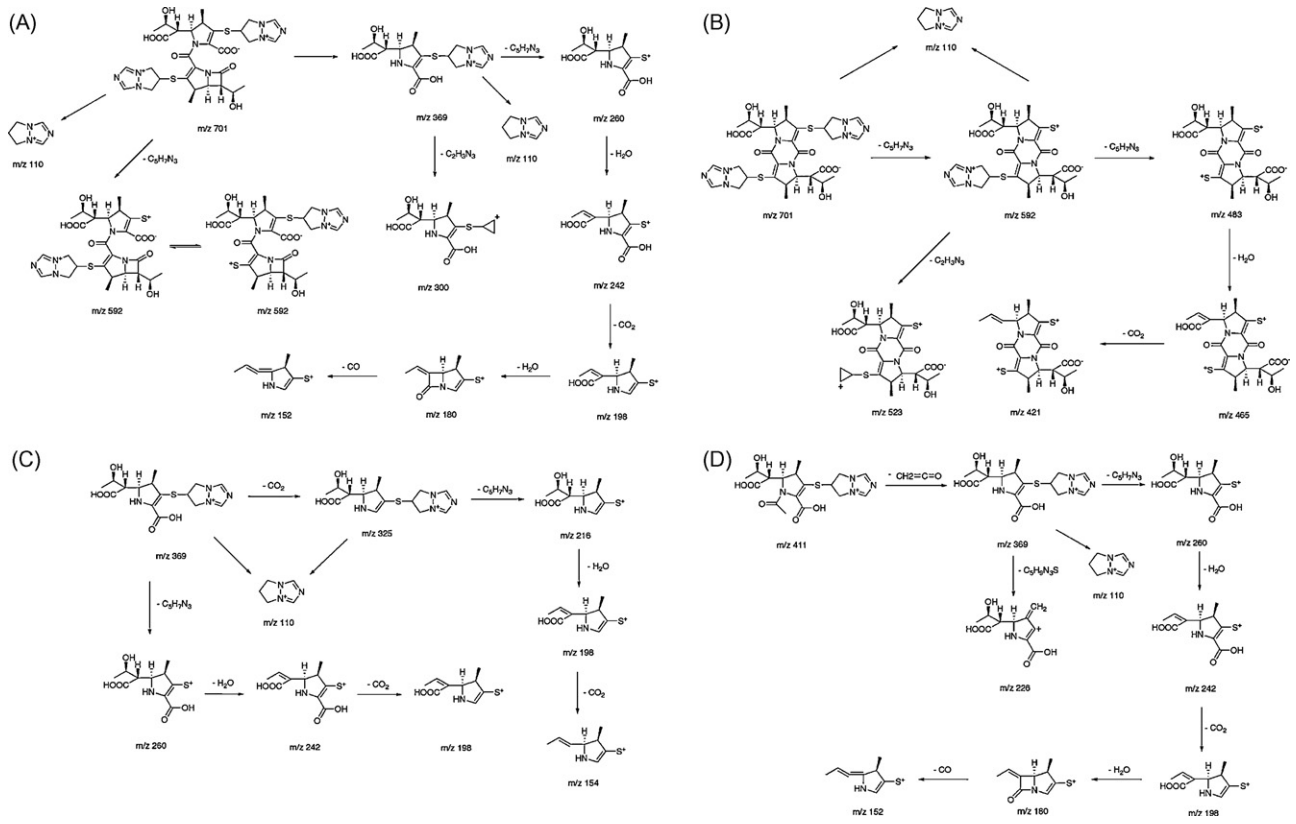

Fig. 5. Proposed fragmentation pathways of biapenem impurities. (A) Dimer A; (B) Dimer B; (C) Impurity I; (D) Impurity III.

lowed a pseudo-first-order degradation in an aqueous solution [9]. The effect of initial concentration on stability of panipenem in an acidic solution was also investigated [10]. Furthermore, Zajac studied general and specific acid–base hydrolysis of ertapenem at various pH and temperatures, using both HPLC and UV methods [11].

In this work, the biapenem aqueous solution was degraded under different conditions varying the concentration pH value, temperature and time. The impurities were detected under the HPLC condition described in Section 2.2. Based on the results proper degradation conditions were selected to obtain material for structural identification of the degradants.

3.2.1. Influence of concentration

The concentration dependence of the formation of degradants is shown in Fig. 2A. The samples in aqueous solution (pH 3.5) were heated at 80 °C in a water bath for 40 min. The plot indicates that Dimer A increased slightly while Dimer B increased sharply till the concentration up to 10 mg/ml. The amount of Dimer B was maintained at 15–18% with concentrations above 20 mg/ml. However, Impurity II formation declined from 31% to 21% with increasing sample concentration. The solution was in supersaturated condition during the degradation process when the concentration exceeded 40 mg/ml.

3.2.2. Influence of pH

The dependence of the formation of biapenem impurities under different pH conditions is shown in Fig. 2B. In this case, 50 mg biapenem was dissolved in 10 ml aqueous solution, heated at 80 °C for 40 min. Acetic acid and aqueous ammonia were used to adjust the pH value from 2.5 to 7.5. It is shown that biapenem was unstable under both acidic and alkaline conditions. When pH was below 3.5, the amount of biapenem decreased sharply. The levels of Impurity I, Impurity II and Dimer B all reached the top at pH 3.0. Furthermore, a significant increase in other degradation products, which were not of interest, was

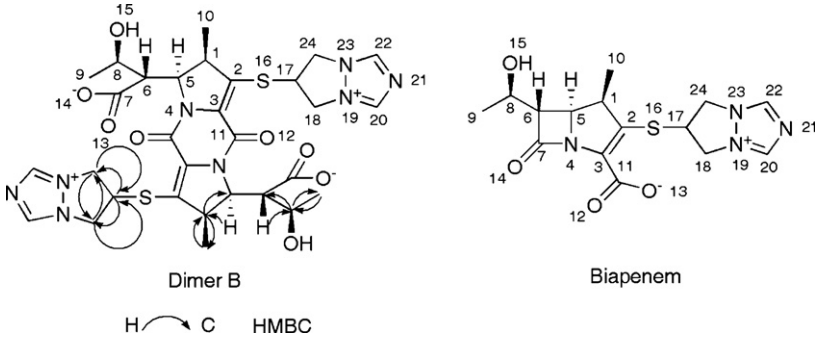

Fig. 6. Molecular structure of Dimer B and biapenem.

Table 1
^{1}H, ^{13}C NMR assignments for biapenem and Dimer B.

Position[a]	^{1}H NMR multiplicity				^{13}C NMR	
	Biapenem		Dimer B		Biapenem	Dimer B
	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	δ (ppm)	δ (ppm)
1	3.39 (1H, m)	–	3.51–3.55 (1H, m)	–	42.5	47.2
2	–	–	–	–	134.0	132.7
3	–	–	–	–	136.2	137.6
5	3.50 (1H, dd)	6.0	5.04–5.09 (1H, m)	–	59.2	61.9
6	2.43 (1H, m)	–	2.43 (1H, d)	10.2	56.1	59.6
7	–	–	–	–	176.7	180.4
8	4.27 (1H, m)	–	4.04–4.07 (1H, m)	–	65.0	69.1
9	1.27 (3H, d)	6.0	1.23 (3H, d)	6.0	20.1	23.1
10	1.23 (3H, d)	9.0	1.46 (3H, d)	7. 5	15.7	13.9
11	–	–	–	–	167.3	157.5
17	4.98 (1H, m)	–	5.04–5.09 (1H, m)	–	45.2	51.9
18, 24	4.68–4.80, 5.01–5.09	–	4.77–4.88, 5.04–5.09 (2H, m)	–	52.5 54.2	55.5 56.1
20, 22	9.01 8.99 (1H, s)	–	9.06 9.05 (1H, s)	–	143. 7 143.6	146.3 146.2

s, singlet; d, doublet; dd, double of doublets; m, multiplet; J, coupling constant.
[a] Refer to Fig. 6 for numbering of biapenem and Dimer B.

observed below pH 3.5. At pH 2.5, these degradation products even surpassed the level of Impurity II. In order to avoid interference with the isolation of Dimer B, the pH was maintained at 3.5.

3.2.3. Influence of temperature

The temperature dependence of the formation of biapenem degradants is shown in Fig. 2C. In this case, 5 mg/ml biapenem aqueous solution (pH 3.5) was stored at various temperatures. The level of the hydrolysis products and other degradation products increased significantly above 40 °C. At temperatures above 60 °C, the amount of Dimer B increased sharply while the formation of Dimer A declined and was almost undetectable at 100 °C.

3.2.4. Influence of time

The time course of the degradation of 50 mg/ml biapenem aqueous solution (pH 3.5) at 80 °C is shown in Fig. 2D. From 0 to 20 min, Dimer A rose to the highest level. After 20 min, the amount of Dimer A declined and finally could not be detected. Furthermore, Dimer B formation got to 25% and maintained at this level after 2 h. Impurity II and other degradation products were formed at an appreciable rate.

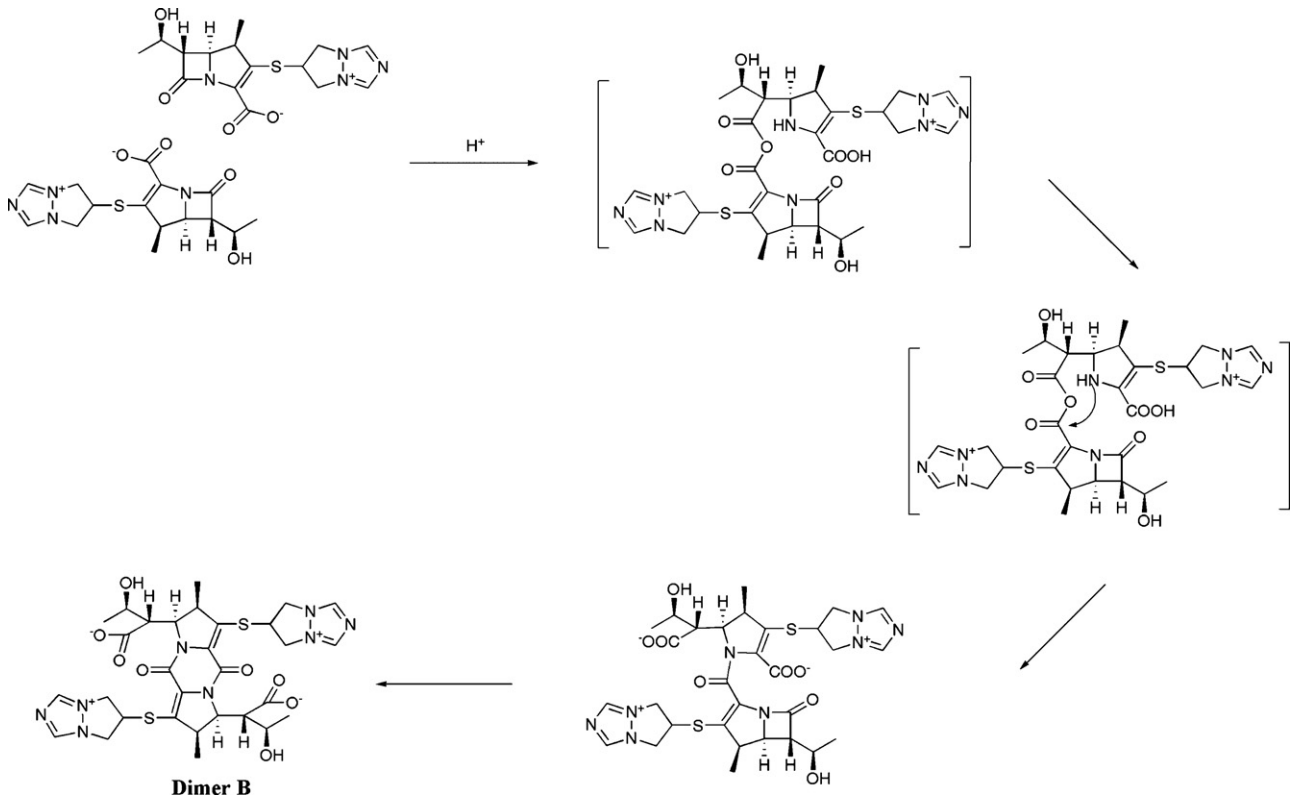

Fig. 7. Proposed formation process of Dimer B.

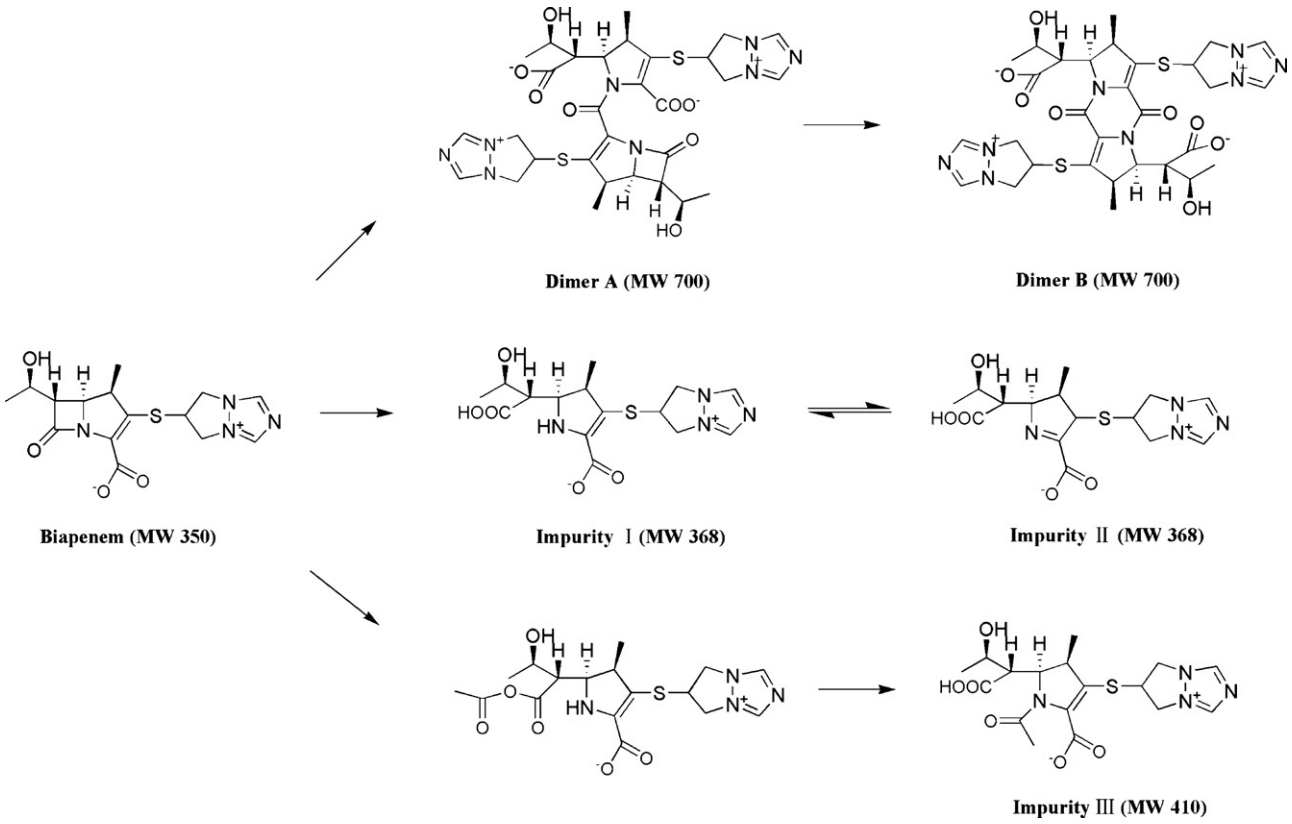

Fig. 8. Proposed mechanism of formation of biapenem impurities.

3.3. LC–MS/MS analyses

The 10 mg/ml biapenem aqueous solution (pH 3.5), heated in a water bath at 80 °C for 5 min, was subjected to LC–MS/MS analysis. The full MS scan was acquired from m/z 200 to 1000. The total ion chromatogram was collected under the conditions described in Section 2.3. The results are shown in Fig. 3. The peaks at RT 2.06, 2.52, 3.82, 5.30, 6.68 and 13.22 min corresponded to Impurity I, Impurity II, Impurity III, biapenem, Dimer A and Dimer B, respectively. The MS^2 experiments were conducted on ions of interest with normalized collision energies between 15 eV and 25 eV, depending on the intensities of product ions induced.

3.4. Structure characterization of Dimer B

The ESI mass spectrum of Dimer B exhibited a molecular ion peak at m/z 701 $[M+H]^+$ in positive ion mode (Fig. 4A), indicating that the molecular weight of Dimer B was 700, which was twice as much as that of biapenem. The ion peak of m/z 723 derived from Na^+ cationization of Dimer B molecule. Furthermore, m/z 701 and m/z 592 were considered to be the precursor ions to obtain MS^2 fragments, respectively. At 25 eV collision energy, m/z 592 and 110 were formed as the major fragments of m/z 701 (Fig. 4B). The MS^2 fragments of m/z 592 were m/z 523, 483, 465, 421 and 110 (Fig. 4C), supposing that the Dimer B structure could most likely contain carboxyl groups and hydroxyl groups. Proposed fragmentation pathway of Dimer B was shown in Fig. 5B.

The HMBC of Dimer B is shown in Fig. 6. The ^1H NMR and ^{13}C NMR data of Dimer B have been compared with that of biapenem in Table 1. Their structures are displayed in Fig. 6. The comparison results indicated that the proton number of Dimer B was twice as many as that of biapenem, except the active ones. However, their carbon numbers were the same. From the above spectral information, it was confirmed that Dimer B was a dimer of biapenem with symmetrical structure.

Based on the literature search and experimental data, it was suggested that at the beginning of the reaction in solution, a ring-opened dimer was formed by consecutive intra-molecular and inter-molecular reaction of β-lactam ring of one biapenem molecule and carboxylic acid group of a second molecule. When the second molecule was hydrolyzed later, another amide bond formed. This is the proposed formation mechanism of Dimer B, similar to the formation process of imipenem dimer [2], shown in Fig. 7.

Finally, Dimer B is characterized as 2,2′-((2R,3S,7R,8S)-1,6-bis(6,7-dihydro-5H-pyrazolo[1,2-a][1,2,4]triazol-4-ium-6-ylthio)-2,7-dimethyl-5,10-dioxo-2,3,5,7,8,10-hexahydrodipyrrolo[1,2-a:1′,2′-d]pyrazine-3,8-diyl)bis((2S,3R,2′S,3′R)-3-hydroxybutanoate), with molecular formula $C_{30}H_{36}N_8O_8S_2$ and molecular weight 700.

3.5. Estimation of the structures of Dimer A, Impurity I, Impurity II and Impurity III

3.5.1. Dimer A

According to the molecular ion peak at m/z 701 $[M+H]^+$ and the sodium adduct m/z 723 $[M+Na]^+$ in positive ion mode, it was indicated that the molecular weight of Dimer A should be 700, which was identical with Dimer B molecular weight.

The major MS^2 fragments ion peaks of m/z 701 were m/z 592, 369 and 110. When m/z 369 was collided with 20 eV collision energy as precursor ion, seven major fragments were formed: m/z 300, 260, 242, 198, 180, 152 and 110. The fragments of m/z 592 and 260 arose from elimination of $C_5H_8N_3$ from m/z 701 and 369, respectively, proving that Dimer A should contain double biapenem structures. Based on the possible fragmentation pathways of Dimer A shown in Fig. 5A, we have supposed that Dimer A would be a ring-opened

dimer of biapenem, similar to imipenem and ertapenem dimers [2,7].

3.5.2. Impurity I and Impurity II

Interestingly, both Impurity I and Impurity II displayed a dominant fragment at m/z 369 in positive ion mode, which was 18 amu more than the molecular ion peak of biapenem. This observation indicated that Impurity I and Impurity II should be the hydrolysis degradation products of biapenem. The main product ions of m/z 369 were m/z 325, 260, 242, 198 and 110. The ion of m/z 325 was fragmented at the collision energy of 20 eV as precursor ion, resulting in the appearance of m/z 216, 198 and 154. Based on the analysis of LC–MS/MS data, it was proved that structures of Impurity I and Impurity II contained $-C_5H_7N_3$, –COOH and –OH parts, similar to that of biapenem. Two main fragmentation pathways of Impurity I were proposed, as shown in Fig. 5C.

Furthermore, Impurity I and Impurity II arose from the opening of β-lactam ring of biapenem molecule. They were a pair of inter-converting tautomeric isomers and could not be isolated well in HPLC, similar to hydrolysis products observed in ertapenem [7].

3.5.3. Impurity III

Impurity III clearly showed a molecular ion peak at m/z 411 $[M+H]^+$ in positive ion mode. The sodium adduct m/z 433 $[M+Na]^+$ was well detectable along with m/z 455 $[M+2Na-H]^+$ and m/z 471 $[M+Na+K-H]^+$, indicating that the molecular weight of Impurity III was 410. The main fragments of m/z 411 were m/z 369 and m/z 260.

To obtain further structural information, m/z 411 and m/z 260 were fragmented at collision energies of 20 eV and 15 eV as precursor ion, respectively. The main product ions of m/z 411 were m/z 369, 260, 226 and 110. The ion at m/z 369 was formed by the loss of 42 from m/z 411, suggesting that the structure of Impurity III contain an acetyl group. MS^2 fragmentation of m/z 260 yielded four major fragments: m/z 242, 198, 180 and 152.

A fragmentation pathway based on the relationships between the MS^2 ions is presented in Fig. 5D. Impurity III was supposed to be the reaction product of biapenem and the acetic acid used in the synthetic process.

4. Conclusion

The impurities in biapenem aqueous solution, including two dimers and three hydrolysis degradation products were studied in this work. One dimer (Dimer B) was isolated, prepared and characterized by preparative HPLC, LC–MS/MS and NMR techniques. Finally, it was confirmed that Dimer B is a dimer of biapenem with symmetrical structure.

In addition, the structures of other impurities were identified by LC–MS/MS. It was supposed that Dimer A should be a ring-opened dimer of biapenem. Impurity I and Impurity II were both ring-opened hydrolysis products of biapenem. They were a pair of inter-converting tautomeric isomers and could not be separated well in HPLC. Furthermore, Impurity III was supposed to be a synthetic process impurity. A possible degradation pathway of biapenem was also suggested (Fig. 8).

Acknowledgements

The constant support and cooperation of the colleagues in Jiangsu Simcere Pharmaceutical R&D Co., Ltd. is highly appreciated. The authors also thank Professor Hang for his useful directions.

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