SEPARATION AND CHARACTERIZATION OF THE IMPURITIES AND ISOMERS IN CEFMENOXIME HYDROCHLORIDE BY HPLC-UV-MS

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SEPARATION AND CHARACTERIZATION OF THE IMPURITIES AND ISOMERS IN CEFMENOXIME HYDROCHLORIDE BY HPLC-UV-MSn

Jian Wang,1 Dan Ruan,2 and Weiguang Shan2
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The twelve impurities and isomers in cefmenoxime hydrochloride were separated and identified by high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MSn). A discipline of mass fragmentation pattern and structure for the E-isomer and Δ3-isomer of oxime cephalosporin antibiotics was presented to distinguish their structures. The column was Alltima C18(250 × 4.6 mm, 5 μm). The mobile phase was water-acetic acid-acetonitrile (85:1:15). In positive mode, full scan LC-MS was first performed in order to obtain the m/z value of the protonated molecules of all detected peaks. LC-MS-MS and LC-MS-MS-MS were then carried out on the compounds of interest. The complete fragmentation patterns of twelve impurities were studied and used to obtain information about the structure of these impurities. The relationship of mass fragmentation pattern and structure for cefmenoxime, E-isomer of cefmenoxime and Δ3-isomer of cefmenoxime was studied. The structures of the twelve impurities and isomers in cefmenoxime hydrochloride were deduced based on the HPLC-MSn data, assisted by the UV spectra and stress testing.

Keywords cefmenoxime hydrochloride, HPLC-ESI-MSn, identification, impurity, isomer, structure

INTRODUCTION

Cefmenoxime Hydrochloride is a third generation cephalosporin antibiotics. It is widely used because of high activity against a large number of both gram-positive and gram-negative micro-organisms and its resistance to β-lactamases. Cefmenoxime hydrochloride is unstable. Some degradation products are produced readily during the production of cefmenoxime hydrochloride and during storage. The clinical effect will be impacted because of the reduction of these impurities’ antibacterial activities and the increase of these impurities’ toxicities. Therefore, it is necessary to characterize and control these impurities.

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The identifications of impurities could be performed by preparative liquid chromatography followed by spectra. It is evident that such a procedure is labor intensive and requires a large amount of degradation material. Moreover, this indirect technique can induce degradation of the compound of interest. Consequently, it is difficult to know whether the identified compound was really an impurity or simply an artifact. This is especially true for β-lactams which have limited stability in organic solvents like methanol or acetonitrile. A direct coupling method is thus more suitable.

The on-line combination of liquid chromatography and mass spectrometry (HPLC-MSn) has developed quickly as an identification tool. HPLC-MSn combines chromatographic strong separation with mass spectrometric strong qualitative advantages. Much structure information can be obtained through LC-MS-MS and LC-MS-MS-MS analysis, which shows great superiority to identify the impurities. Structural identifications of the impurities in cephalosporin antibiotics have been reported, but structural identification of the impurities in cefmenoxime hydrochloride has not been reported. The aim of this study was to separate and characterize the impurities in Cefmenoxime Hydrochloride by HPLC-ESI-MSn, assisted by the UV spectra and stress testing. Twelve impurities and isomers in cefmenoxime hydrochloride drug substance were separated and characterized, and a complete structure was proposed for twelve of these.

The relationship of mass fragmentation pattern and structure for cefmenoxime, E-isomer of cefmenoxime, and Δ3-isomer of cefmenoxime was studied. The relationship of mass fragmentation pattern and structure for deacetylcefotaxime (a impurity), E-isomer of deacetylcefotaxime, and Δ3-isomer of deacetylcefotaxime was studied. A discipline of mass fragmentation pattern and structure for the E-isomer and Δ3-isomer of oxime cephalosporin antibiotics was presented to distinguish their structures.

**EXPERIMENTAL**

**Chemicals and Reagents**

Cefmenoxime hydrochloride reference substance and drug substance (batch numbers: 101217) and intermediates were provided by Zhejiang Jinhua Kangenbei Pharmaceutical Co. Ltd. (Jinhua, China). Acetic acid and acetonitrile were chromatographic grade.

**Instrumentation**

**LC Apparatus**

An Agilent 1260 series liquid chromatography (LC) system equipped with a binary pump and a UV detector was connected to an Agilent
G1313A autosampler. Chromatographic separation was carried out at room temperature using an Alltima C18 analytical column (250 × 4.6 mm, 5 μm). The mobile phase consisted of water-acetic acid-acetonitrile (85:1:15). The flow rate was 0.8 mL/min. Column oven temperature was maintained at 30°C and injection volume was 20 μL.

**Mass Spectrometry**

LC-MS experiment was carried out on an AB SCIEX 4000 Q TRAP composite triple quadrupole/linear ion trap tandem mass spectrometer. The column effluent was split using a zero-dead-volume “T” connector, with approximately one quarter of the flow being fed to the mass spectrometer. The MSD was equipped with an ESI source. The ionization mode was positive. The interface and MSD parameters were as follows: nebulizer pressure (40 p.s.i.), dry gas pressure (40 p.s.i.), curtain gas pressure (10 p.s.i.), dry gas temperature (650°C), spray capillary voltage (5500V), CAD: high, DP: 80 V, EP: 20 V, CE: 45 V.

All data were acquired and processed by Analyst software.

**Sample Solution Preparation**

Dissolve adequate cefmenoxime hydrochloride drug substance with water-acetic acid-acetonitrile (70:1:30) to make the solution of 1.0 mg/mL.

**RESULTS AND DISCUSSION**

**Selection of Chromatographic Conditions**

In order to permit the use of liquid chromatography-mass spectrometry analysis, acid in the mobile phase must be a volatile acid and its concentration should be as low as possible. By testing, we found that the retention time of cefmenoxime was appropriate, and cefmenoxime could be completely separated from intermediates, excipient, and degradation products when using water-acetic acid-acetonitrile (85:1:15) as the mobile phase. The chromatogram of cefmenoxime hydrochloride drug substance is shown in Figure 1.

**Stress Testing and Results**

Samples were stored under relevant stress conditions (light, heat, acid/base hydrolysis, and oxidation, respectively). The results are shown in Table 1.
Acid Degradation

The 20 mg of cefmenoxime hydrochloride was introduced into a test tube. Then, 0.5 mL of 1 M HCl was added and heated for 30 min at 60°C. After 30 min, the drug treated with 1 M HCl was neutralized with 1 M NaOH and diluted with water-acetic acid-acetonitrile (70:1:30) to 20 mL.

Basic Degradation

The 20 mg of cefmenoxime hydrochloride was introduced into a test tube. Then, 0.5 mL of 1 M NaOH was added and heated for 30 min at

TABLE 1 Change of the Impurity Content Under the Stress Condition and Natural Degradation

<table>
<thead>
<tr>
<th>No.</th>
<th>Basic Damage</th>
<th>Acid Damage</th>
<th>High Temperature Damage</th>
<th>Ultraviolet Light Damage</th>
<th>Oxidative Damage</th>
<th>High Temperature Damage of Solution</th>
<th>Ultraviolet Light Damage of Solution</th>
<th>Natural Degradation of One Week</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>II</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
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</tr>
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<td>III</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
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</tr>
<tr>
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<td>-</td>
<td>+</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>IX</td>
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<td>-</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>X</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XI</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XII</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: –, meant that the impurity content had no change.
+ , meant that the impurity content increased.
++ , meant that the impurity content increased obviously.
100°C. After 30 min, the drug treated with 1 M NaOH was neutralized with 1 M HCl and diluted with water-acetic acid-acetonitrile (70:1:30) to 20 mL.

**Oxidative Degradation**

The 20 mg of cefmenoxime hydrochloride was introduced into a test tube. Then, 0.2 mL of 3% H₂O₂ was added and heated for 30 min at 60°C. After 30 min, the drug treated with 3% H₂O₂ was diluted with water-acetic acid-acetonitrile (70:1:30) to 20 mL.

**Heat Degradation**

The 20 mg of cefmenoxime hydrochloride was heated for 3 hr at 105°C. After 3 hr, the drug was diluted with water-acetic acid-acetonitrile (70:1:30) to 20 mL.

**Photolytic Degradation**

The 20 mg of cefmenoxime hydrochloride was introduced into a Petri dish which was exposed to ultraviolet light at 254 nm for 24 hr. After 24 hr, the drug was diluted with water-acetic acid-acetonitrile (70:1:30) to 20 mL.

**Solution Heat Degradation**

Dissolve adequate cefmenoxime hydrochloride drug substance with water-acetonitrile (70:30) to make the solution of 1.0 mg/mL. This solution was heated for 1 hr at 60°C.

**Solution Photolytic Degradation**

Dissolve adequate cefmenoxime hydrochloride drug substance with water-acetonitrile (70:30) to make the solution of 1.0 mg/mL. This solution is exposed to ultraviolet light at 254 nm for 24 hr.

**Selection of MS Conditions**

APCI and ESI are two common used LC/MS ion source, APCI is more suitable to the analysis of neutral or small polar compounds, while ESI is more suitable to the analysis of polar, thermo-labile alkaline, or acidic compounds. According to the structural characteristic of cefmenoxime hydrochloride and its impurities, ESI was selected to analyze the impurities in cefmenoxime hydrochloride. Compared with negative mode, the impurities in cefmenoxime hydrochloride had higher MS response and sensitivity in positive mode. In order to obtain the proper fragment ions, various interface and MSD parameters were tested. The results showed that the interface and MSD parameters in Mass Spectrometry were optimal. Full scan LC-MS
Structure Elucidation

**Impurity IX, Impurity XI, and Impurity XII**

Impurity IX, impurity XI, and impurity XII exhibited the same molecular species [M +H]⁺ at m/z 512, [M +Na]⁺ at m/z 534 and [M +K]⁺ at m/z 550 in LC-MS analysis, indicating the same molecular mass of the three impurities as 511, which is equivalent to the mass of cefmenoxime. Therefore, they were suggested as isomers of cefmenoxime. The MS-MS spectra and MS-MS-MS spectra is presented in Figure 2.

Figure 3 presents the following fragmentation sequence of cefmenoxime: a loss of a 1-methyl-1H-tetrazole-5-thiol with the formation of the ion at m/z 396; a loss of a neutral of 44 Da (CO₂) leading to the fragment at m/z 352; a loss of 28 Da (CO) leading to the ion at m/z 324. The loss of CO before CO₂ also happened from the ion at m/z 396 to give the ions at m/z 368 and 324, respectively. The subsequent fragmentation involved the loss of 157 leading to the ion at m/z 167. Another fragmentation pathway concerned the cleavage of the β-lactam ring from the ion at m/z 396 conducting to the fragmentation at m/z 241.

The content of impurity XII increased obviously after cefmenoxime hydrochloride was introduced into a Petri dish which was exposed to ultraviolet light at 254 nm for 24 hr. That E-isomers of several oxime cephalosporin antibiotics formed by cis-trans isomerization of the oxime function when exposed to ultraviolet light have been reported,[3] suggesting the impurity XII as E-isomer of cefmenoxime. It was supported further by the MS-MS spectrum and MS-MS-MS spectrum of the literature[2] and the impurity XII. It concerned the loss of CH₃O- from the ion at m/z 324 leading to the fragment at m/z 293. The fragmentation at m/z 293 in cefmenoxime (Z) had low abundance because the methoxy group of the oxime with amine close to it formed N-O hydrogen bonding. The formed structure was stable, and the methoxy group of the oxime was not easily lost. However, the fragmentation at m/z 293 in the impurity XII (E-isomer of cefmenoxime) was abundant because the methoxy group of the oxime function with amine away from it did not easily form N-O hydrogen bonding and the methoxy group of the oxime function was easily lost. Figure 4 presents the fragmentation sequence of the impurity XII.
### TABLE 2  Mass Spectra Results of Twelve Impurities in Cefmenoxime Hydrochloride

<table>
<thead>
<tr>
<th>Impurity</th>
<th>t_R (min)</th>
<th>[M+H]^+ (m/z)</th>
<th>Major MS/MS Fragmentation Ions (m/z)</th>
<th>Major MS/MS Fragmentation Ions (m/z)</th>
<th>Proposed Structure of the Impurity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3.1</td>
<td>396 352 321 247 230</td>
<td>352—321 230</td>
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<td><img src="image" alt="Proposed Structure for Impurity I" /></td>
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<td>II</td>
<td>3.5</td>
<td>243 126</td>
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<td></td>
<td><img src="image" alt="Proposed Structure for Impurity II" /></td>
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<td>III</td>
<td>3.7</td>
<td>414 241 197 126</td>
<td>241—197 197—166</td>
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<td><img src="image" alt="Proposed Structure for Impurity III" /></td>
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<tr>
<td>IV</td>
<td>4.3</td>
<td>414 285 241 227 200</td>
<td>285—254 257 225</td>
<td></td>
<td><img src="image" alt="Proposed Structure for Impurity IV" /></td>
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</table>

(Continued)
<table>
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<tr>
<th>Impurity</th>
<th>$t_R$ (min)</th>
<th>[M+H]$^+$ (m/z)</th>
<th>Major MS/MS Fragmentation Ions (m/z)</th>
<th>Major MS/MS Fragmentation Ions (m/z)</th>
<th>Proposed Structure of the Impurity</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>4.9</td>
<td>414</td>
<td>285 241 227</td>
<td>285→257 225</td>
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<tr>
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<td>5.3</td>
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<tr>
<td>VII</td>
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<td>117</td>
<td>99 74</td>
<td></td>
<td><img src="image3" alt="Proposed Structure of VII" /></td>
</tr>
<tr>
<td>VII</td>
<td>9.3</td>
<td>396</td>
<td>241 227 200</td>
<td>227→200</td>
<td><img src="image4" alt="Proposed Structure of VII" /></td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>IX</td>
<td>11.4</td>
<td>512</td>
<td>396</td>
<td>352</td>
<td>241</td>
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<tr>
<td></td>
<td>201</td>
<td>152</td>
<td>197</td>
<td>166</td>
<td></td>
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<td>241</td>
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</table>

![Image of IX molecule]

<table>
<thead>
<tr>
<th>X</th>
<th>12.2</th>
<th>398</th>
<th>285</th>
<th>241</th>
<th>227</th>
<th>241→167</th>
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<td>117</td>
<td>167</td>
<td>117</td>
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![Image of X molecule]

<table>
<thead>
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<th>XI</th>
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<th>512</th>
<th>396</th>
<th>368</th>
<th>352</th>
<th>396→324</th>
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<td>241</td>
<td>211</td>
<td>167</td>
<td>211</td>
<td>167</td>
<td>117</td>
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</table>

![Image of XI molecule]

<table>
<thead>
<tr>
<th>XII</th>
<th>27.2</th>
<th>512</th>
<th>396</th>
<th>324</th>
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<th>396→352</th>
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<td>126</td>
<td>112</td>
<td></td>
<td>241</td>
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</tr>
</tbody>
</table>

![Image of XII molecule]
The ultraviolet absorption of the impurity IX shifted toward short wavelength compared with cefmenoxime ($\Delta^2$), which indicated its conjugated system was reduced. This suggested the impurity IX as $\Delta^3$-isomer of cefmenoxime. It was supported further by the MS-MS spectrum and MS-MS-MS spectrum of the literature[1] and the impurity IX. The loss of CO from the ion at $m/z$ 396 provided the ions at $m/z$ 368. However, the absence of the fragment ion at $m/z$ 368 in the impurity IX, attributed for cefmenoxime, was not detected in this case. The subsequent fragmentation ions at $m/z$ 277 and at $m/z$ 167 in the impurity IX attributed to cefmenoxime was also not detected. This was because different positions of double bonds in $\Delta^2/\Delta^3$-cefmenoxime caused electron density different in the ring. Figure 5 presents the fragmentation sequence of the impurity IX.
The fragmentation ion patterns of the impurity XI and cefmenoxime were very similar suggesting that it was a structural analogue of the main drug. The content of impurity XI increased obviously in the basic condition. The 7-epimer of several cephalosporin antibiotics produced in basic condition have been reported,\cite{3} suggesting the impurity XI as 7-epimer of cefmenoxime.

**Impurity III, Impurity IV, and Impurity V**

The impurity III, impurity IV, and impurity V exhibited the same protonated molecule [M+H]$^+$ at $m/z$ 414 in LC-MS analysis, indicating the same molecular mass of the three impurities as 413. The molecular masses of them were equivalent to the mass of deacetylcefotaxime, an impurity of cefotaxime listed in the British Pharmacopoeia, which were, therefore, suggested as deacetylcefotaxime and isomers of deacetylcefotaxime. The MS-MS spectra and MS-MS-MS spectra of the impurity III, impurity IV,
and impurity V verified that their structures were in agreement with deacetylcefotaxime. Figure 6 presents the proposed fragmentation patterns of impurity IV, impurity IV, and impurity V.

The fragmentation pathways of impurity IV and impurity V concerned the cleavage of the β-lactam ring conducting to the fragmentation at m/z 285 and at m/z 241. The content of impurity IV increased, but the content of impurity V did not change after cefmenoxime hydrochloride was introduced into a Petri dish that was exposed to ultraviolet light at 254 nm for 24 hr, suggesting the impurity IV as E-isomer, and the impurity V as Z-isomer. It was supported further by the MS-MS spectra and MS-MS-MS spectra of the literature, the impurity IV and the impurity V. It concerned the loss of CH$_3$O- from the ion at m/z 285 leading to the fragmentation at m/z 254. The fragmentation at m/z 254 in the impurity V (Z-isomer) had low abundance because the methoxy group of the oxime with amine close to it formed N-O hydrogen bonding. The formed structure was stable, and the methoxy group of the oxime was not easily lost. On the contrary, the fragmentation at m/z 254 in the impurity IV (E-isomer) was abundant because the methoxy group of the oxime function with amine away from it did not easily formed N-O hydrogen bonding and the methoxy group of the oxime function was easily lost.

The ultraviolet absorption of the impurity III shifted toward short wavelength compared with impurity IV and impurity V ($\Delta^2$), which indicated its conjugated system reduced. This suggested the impurity III as $\Delta^3$-isomer. It was supported further by the MS-MS spectrum and MS-MS-MS spectrum.
FIGURE 5 Proposed fragmentation pathways of the impurity IX(Δ3 Cefmenxime).

FIGURE 6 Proposed fragmentation patterns of impurity III (a), impurity IV(b), and impurity V(c).
of the impurity III. The fragmentation pattern of impurity III was different with the fragmentation patterns of impurity IV and impurity V. The absence of the fragmentation at \( m/z \) 285 in the impurity III, attributed for impurity IV and impurity V, was not detected in this case. This was because different positions of double bond in \( \Delta^2/\Delta^3 \)-isomers. The main MS-MS fragmentation of impurity III was at \( m/z \) 241. The subsequent fragmentation involved the loss of COOH from the ion at \( m/z \) 241 leading to the ion at \( m/z \) 197.

**Impurity X**

The impurity X exhibited the protonated molecule \([M + H]^+\) at \( m/z \) 398 in LC-MS analysis, indicating the molecular weight of the impurity as 397. Its structure is in agreement with an impurity of cefotaxime listed in British Pharmacopoeia, which was named as deacetoxycefo-taxime. The HPLC retention time and MS-MS spectra of impurity X and deacetoxycefo-taxime were identical to verify an identity between them. Figure 7 presents the proposed fragmentation patterns of impurity X.

**Impurity I and Impurity VIII**

The impurity I and impurity VIII exhibited the same protonated molecule \([M + H]^+\) at \( m/z \) 396 in LC-MS analysis, indicating the same molecular mass of the two impurities as 395. Therefore, they were suggested as isomers. The proposed fragmentation sequences of the impurity I and impurity VIII are presented in Figure 8 and Figure 9.

The content of impurity I increased, but the content of impurity VIII did not change after cefmenoxime hydrochloride solution is exposed to ultraviolet light at 254 nm for 24 hr,\(^5\) suggesting the impurity I as E-isomer, and the impurity VIII as Z-isomer. It was supported further by the MS-MS spectra and MS-MS-MS spectra of the literature,\(^2\) the impurity I and the impurity VIII. It concerned the loss of CH\(_3\)O- from the ion at \( m/z \) 352 leading to the fragmentation at \( m/z \) 321. The fragmentation at \( m/z \) 321 in the impurity VIII (Z-isomer) had low abundance because the methoxy group of the oxime with amine close to it formed N-O hydrogen bonding. The formed structure was stable, and the methoxy group of the oxime function

![Diagram of impurity X](image-url)
was not easily lost. On the contrary, the fragmentation at \( m/z \) 321 in the impurity I (E-isomer) was abundant because the methoxy group of the oxime function with amine away from it did not easily form N-O hydrogen bonding and the methoxy group of the oxime function was easily lost.

**Impurity II**

The impurity II exhibited the molecular species \([M+H]^+\) at \( m/z \) 243 and \([M+Na]^+\) at \( m/z \) 265 in LC-MS analysis, indicating the molecular mass of the impurity as 242. Its structure was deduced based on the HPLC-MS\(^n\) data. Figure 10 presents the proposed fragmentation sequence of impurity II.

**Impurity VII**

The impurity VII exhibited the protonated molecule \([M+H]^+\) at \( m/z \) 117 in LC-MS analysis, indicating the molecular mass of the impurity as 116. Its structure is in agreement with an intermediate, which was named
as 1-methyl-1H-tetrazole-5-thiol (MMTZ). The HPLC retention time and MS-MS spectra of impurity VII and MMTZ were identical to verify an identity between them.

**Impurity VI**

The impurity VI exhibited the molecular species \([M + H]^+\) at \(m/z\) 528, \([M + Na]^+\) at \(m/z\) 550 and \([M + K]^+\) at \(m/z\) 566 in LC-MS analysis, indicating the molecular mass of the impurity as 527, which is 16 Da more than that of cefmenoxime. The content of impurity VI increased obviously after cefmenoxime hydrochloride was damaged by oxidation, suggesting the impurity VI as 5S-oxide of cefmenoxime. Figure 11 presents the proposed fragmentation patterns of impurity VI.

**CONCLUSIONS**

This study was to separate and characterize the impurities in Cefmenoxime Hydrochloride by HPLC-ESI-MS\(^n\), assisted by the UV spectra and stress testing. Twelve impurities and isomers in cefmenoxime hydrochloride drug substance were separated and characterized, and their mass spectrometry splitting rules were studied. A discipline of mass fragmentation pattern and structure for the \(E\)-isomer and \(\Delta^3\)-isomer of oxime cephalosporin antibiotics was presented to distinguish their structures.

Cefmenoxime hydrochloride is unstable. Some degradation products are produced readily during the production of cefmenoxime hydrochloride.
and during storage. These were found to be structural analogues of the main compound as suggested by the close $m/z$ values of the protonated molecules $[\text{M+H}]^+$ and the similar main fragmentation patterns. Furthermore, the results suggested that some stereoisomers could be produced by degradation.

REFERENCES