Determination and Characterization of Two Degradant Impurities in Bendamustine Hydrochloride Drug Product

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Abstract

Bendamustine hydrochloride is an alkylating antitumor agent with a good efficacy in the treatment of chronic lymphocytic leukemia (CLL) and B-cell non-Hodgkin’s lymphoma (B-NHL). Under the stressed conditions, two degradant impurities in bendamustine hydrochloride drug product were detected by high-performance liquid chromatography. These two degradant impurities were isolated from preparative liquid chromatography, and were further characterized using Q-TOF/MS and nuclear magnetic resonance (NMR). Based on the MS and NMR spectral data, they were characterized as 4-[5-(2-chloroethylamino)-1-methyl-1H-benzoimidazol-2-yl] butyric acid hydrochloride (impurity-A) and 4-{5-[[2-(4-{5-[(bis-(2-chloroethyl) amino)-1-methyl-1H-benzoimidazol-2-yl]-butyryloxy}-ethyl]-2-chloroethy]lamino]-1-methyl-3a, 7a-dihydro-1H-benzoimidazol-2-yl) butyric acid hydrochloride (impurity-B). Isolation, structural elucidation of these two impurities by spectral data (Q-TOF/MS, 1H NMR, 13C NMR, D2O exchange NMR and two-dimensional NMR) and the probable formation mechanism of the impurities were discussed.

Introduction

Bendamustine hydrochloride (BMH), chemically 4-{5-[bis-(2-chloroethyl) amino]-1-methyl-1H-benzoimidazol-2-yl} butyric acid hydrochloride, is an anticancer agent as single-agent therapy (1, 2) or in combination with other antineoplastic drugs (3–7). BMH shows a good efficacy in the treatment of various tumoral diseases (8–10) such as chronic lymphocytic leukemia (CLL) and B-cell non-Hodgkin’s lymphoma (B-NHL) and thus BMH was approved by the US Food and Drug Administration for the treatment of CLL and B-NHL.

Impurities present in drugs are critical to the quality and safety, and should be identified and characterized to ensure the quality and safety of pharmaceutical products. Impurities and degradation products could arise from the manufacturing process and the stressed storage conditions, under the influence of temperature, humidity and photolysis. To simulate the impurities and degradation products which may be formed during shelf-life period, stress testing including thermal stressed testing, humidity stressed testing and photostability testing of pharmaceutical products must be conducted (11). Recently, there has been series of studies (12–15) published for characterization of potential drug impurities in drug substance or drug product using ICH Q3A (2) by employing modern sophisticated analytical methods.

The methods for determination of BMH and its γ-hydroxyl, N-desmethyl, or 2-fold hydrolysis metabolites in biological samples were developed and reported (16–18), but to date, there is only limited data available in the investigation of the impurities and degradation products in BMH or its dosage forms (19). For the stress testing of BMH drug product, we found that two impurities (impurity-A and impurity-B) were formed under the condition of photostability testing and thermal stressed testing, respectively. To our best knowledge, the formation, identification and characterization of the two impurities have not been reported previously in the literature. The aim of this study was to develop a high-performance liquid chromatography (HPLC) method to determine and characterize the impurities and
degradation products in BMH drug product. These two impurities were identified by Q-TOF/MS, one-dimensional (1D) nuclear magnetic resonance (NMR) and two-dimensional (2D) NMR. Plausible mechanisms for the formation have also been proposed in this study.

**Experimental**

**Chemicals, reagents and samples**

The investigated samples of BMH and impurities were prepared in Jiangsu Simcere Pharmaceutical Group Ltd, China (Nanjing, China). Acetonitrile (TEDIA, HPLC grade, Ohio, USA) and water purified by Milli-Q system (Millipore, USA) were used for the preparation of samples and mobile phases. Sodium dihydrogen phosphate (Aladdin, HPLC grade, Shanghai, China) and phosphoric acid (TEDIA, HPLC grade, Ohio, USA) were used for HPLC analysis. Methanol (Merck, HPLC grade, Germany) was used for MS experiments and trifluoroacetic acid (Aladdin, 98%, w/w, AR grade, Shanghai, China) was used both for MS and preparative isolation experiments. Solvent for NMR, DMSO-d6 (Isotech, 99.9 Atom%D, with 0.05% TMS, v/v) was purchased from Sigma–Aldrich Trading Co., Ltd (Shanghai, China). All the chemicals were used as received.

**Stress testing of BMH drug product**

Stress testing of the drug substance is usually used as a tool to identify the likely degradation products, which can in turn help investigate the degradation pathways and the intrinsic stability of the molecule, and validate the stability indicating power of the analytical procedures used (20). To examine the conditions responsible for the formation of the degradation products, stress testing was performed on the BMH drug product.

**Thermal stressed testing**

BMH drug product was kept in a Petri dish under the condition of 60°C for 10 days.

**Humidity stressed testing**

BMH drug product was kept in a Petri dish under the condition of 92.5% relative humidity (RH) for 10 days.

**Photostability testing**

BMH drug product was kept in a Petri dish under the condition of 4,500 lx ± 500 lx for 10 days.

**HPLC instrumentation and methods for analysis**

Chromatographic separations were performed on an Agilent HPLC system (Agilent Technologies, USA) equipped with a G1322A 1200 vacuum degasser, an Agilent 1200 quaternary pump (model G1311A), an Agilent 1200 autosampler (model G1367B), a G1330B 1290 thermostat, a G1316A 1260 temperature controlled column compartment and a G1315D 1260 diode array UV detector. The ChemStation software (Rev.B.04.02 [96]) supplied by Agilent Technologies was used for the signal acquisition and peak integration.

The analysis was carried out on a Gemini C18 column (150 × 4.6 mm, 3 μm; Phenomenex, USA). The mobile phase was a mixture of solvent A and solvent B at the proportion of 70:30 (v/v). The solvent A was sodium dihydrogen phosphate (10 mM) and its pH was adjusted to 2.6 using phosphoric acid, while solvent B was acetonitrile. The flow rate of the mobile phase was 1.0 mL/min and the column was thermostated at a temperature of 25°C. The injection volume was 10 μL and the analytes were monitored at the UV wavelength of 233 nm.

**Sample preparation for HPLC analysis**

BMH drug product and its stress testing samples were separately dissolved in mobile phase (see section “HPLC instrumentation and methods for analysis”) to obtain a concentration of 0.1 mg/mL for analytical HPLC. The mobile phase used as solvent was reserved in ice-water bath to keep cold and the samples were analyzed by HPLC method described in section “HPLC instrumentation and methods for analysis” within 1 h.

**Instrumentation and LC-MS conditions**

MS experiments were performed on an Agilent G6410 Mass Spectrometer (Agilent Technologies, USA) with a triple quadrupole analyzer coupled to an Agilent HPLC 1200 system (Agilent Technologies, USA). LC separation was performed on an Agilent SB-C18 column (150 × 4.6 mm, 3.5 μm; Agilent Technologies, USA) using 0.2 mL/min flow rate and the mobile phase consisting of water (pH* adjusted to 2.6 with trifluoroacetic acid) and methanol at the proportion of 70:30 (v/v). For the ionization of the analytes, a electrospray ionization (ESI) ion source was operated in the positive ion mode (desolvation temperature 400°C, capillary temperature 300°C, discharge current 4 μA and tube lens voltage 40 V).

**Sample preparation for LC-MS**

The thermal stressed testing sample and the photostability testing sample were separately prepared by mixing water and methanol in the ratio of 70:30 (v/v) and the concentration of each sample was 0.5 mg/mL.

**Chromatographic conditions for preparative isolation**

Preparative isolation of impurities A and B was performed on an Agilent 1200 series preparative HPLC system which was equipped with a diode array detector and ChemStation software. A SunFire™ C18 OBD™ column (50 × 250 mm, 5 μm; Waters, USA) was used for preparative isolation work. The flow rate of the mobile phase trifluoroacetic acid (0.1%, v/v)–acetonitrile (70:30, v/v) was 5.0 mL/min and the analytes were monitored at the UV wavelength of 233 nm. A mixture of water and acetonitrile in the proportion of 70:30 (v/v) was used as solvent for sample preparation. The major impurity containing fractions were collected and solvent was removed by vacuum freeze drying to obtain the impurity-A (HPLC purity: 94.8%) and impurity-B (HPLC purity: 92.8%) as white solid.

**High-resolution Q-TOF/MS conditions**

High-resolution mass spectra were recorded on Waters Q-TOF Micro™ mass spectrometer (Waters micro mass, Manchester, UK). Detection of the ions was performed in ESI, both positive and negative ion modes. A mixture of water and methanol at the proportion of 70:30 (v/v) was used as diluent for sample preparation. The samples were infused directly using a syringe at a concentration of 5.0 μg/mL.

**NMR spectroscopy**

The 1H NMR, 13C NMR, D2O exchange and 2D NMR including DEPT (Distortionless Enhancement by Polarization Transfer), 1H–1H COSY (correlated spectroscopy), HSQC (Heteronuclear Single Quantum Coherence Spectroscopy) and HMBC (Heteronuclear Multibond Coherence Spectroscopy) NMR experiments were performed.
**Table I** Comparative $^1$H and $^{13}$C NMR Assignments for Bendamustine Hydrochloride, Impurity-A and Impurity-B

<table>
<thead>
<tr>
<th>Position</th>
<th>BMH $^1$H (ppm)/multiplicity (J, Hz)</th>
<th>$^{13}$C (ppm)</th>
<th>Impurity-A $^1$H (ppm)/multiplicity (J, Hz)</th>
<th>$^{13}$C (ppm)</th>
<th>Impurity-B $^1$H (ppm)/multiplicity (J, Hz)</th>
<th>$^{13}$C (ppm)</th>
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<td>1</td>
<td>7.74/1H/d (9.2)</td>
<td>113.35</td>
<td>7.71/1H/d (9.0)</td>
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<td>112.35</td>
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<td>115.10</td>
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s, singlet; d, doublet; dd, doublet of a doublet; t, triplet; br, broad; m, multiplet.
Results
Detection of impurities and the stress testing results

The stress testing samples of BMH drug product were analyzed by HPLC method as described in section “HPLC instrumentation and methods for analysis”. The analysis revealed the presence of two peaks marked as impurity-A at relative retention time (RRT) of 0.39 and impurity-B at RRT of 1.37 in the chromatogram. Impurity-A and impurity-B are the target impurities, while impurity-C was a known impurity (19) that existed both in BMH and its drug product. Under thermal stressed testing, impurity-B increased from 0.06 to 0.32%. In photostability testing, impurity-A increased from 0.00 to 0.18%. In humidity stressed testing, there was no significant degradation. A typical HPLC chromatogram of photostability testing sample is shown in Figure 1.

Structural elucidation of impurities

The \(^1\)H and \(^{13}\)C NMR chemical shift values of BMH, impurity-A and impurity-B are listed in Table I. The HSQC, HMB and COSY spectra of impurity-A and impurity-B are summarized in Table II. The chemical structures of BMH, impurity-A, impurity-B and numbering scheme for NMR are shown in Figure 2.

Structural elucidation of impurity-A

The molecular ion peak of m/z 296 [M+H] \(^+\) by LC-MS analysis indicated a mass of 295, which was 62 amu less than that of bendamustine. This indicates that BMH may lose a CHCl = CH \(_2\) to produce impurity-A. The Q-TOF/MS spectrum of impurity-A showed molecule ion peak at 296.1168 [M+H] \(^+\) in positive ion mode and 294.1011 [M-H] \(^-\) in negative ion mode, suggesting that the elemental composition of impurity-A in molecular form is C\(_{14}\)H\(_{18}\)N\(_3\)O\(_2\)Cl.

In \(^1\)H-NMR spectrum, the proton signals appearing at δ 6.84 ppm (2H, br) were exchangeable in negative ion mode, suggesting that the elemental composition of impurity-A in molecular form is C\(_{14}\)H\(_{18}\)N\(_3\)O\(_2\)Cl.

In negative ion mode, the proton signals appearing at δ 6.84 ppm were H-14 and H-15. The proton signals appearing at δ 7.14 and 7.07 ppm were H-9 and the proton signals appearing at δ 15.00 ppm (1H, br) were exchangeable. The proton signals appearing at δ 17.31 and 17.30 ppm (2H, br) were exchangeable in negative ion mode, suggesting that the elemental composition of impurity-A in molecular form is C\(_{14}\)H\(_{18}\)N\(_3\)O\(_2\)Cl.

COSY 1H-1H (ppm) HSQC 1H-13C (ppm) HMBC 1H-13C (ppm)

| Impurity-A | 7.71, 7.07–7.14 | 7.14–115.10 | 2.40, 2.02–173.64 |
| Impurity-B | 7.33, 7.72, 6.94, 6.93–7.12 | 7.73–113.40 | 2.41, 2.03–173.11 |
|            | 3.51–3.80      | 3.89, 3.16, 2.02–151.77 |
|            | 3.16, 2.40–2.02 | 3.89, 3.16, 2.02–151.77 |

Table II Correlations Observed in COSY, HSQC and HMBC Spectra of Impurity-A and Impurity-B

<table>
<thead>
<tr>
<th>COSY 1H-1H (ppm)</th>
<th>HSQC 1H-13C (ppm)</th>
<th>HMBC 1H-13C (ppm)</th>
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<tr>
<td>Impurity-A</td>
<td>7.71, 7.07–7.14</td>
<td>7.14–115.10</td>
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<td></td>
<td>3.51–3.80</td>
<td>3.89, 3.16, 2.02–151.77</td>
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<td>3.16, 2.40–2.02</td>
<td>3.89, 3.16, 2.02–151.77</td>
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<tr>
<td>Impurity-B</td>
<td>7.73, 7.72, 6.94, 6.93–7.12</td>
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Structural elucidation of impurity-B

The 1H and \(^{13}\)C NMR chemical shift values of BMH, impurity-A and impurity-B are listed in Table I. The HSQC, HMB and COSY spectra of impurity-A and impurity-B are summarized in Table II. The chemical structures of BMH, impurity-A, impurity-B and numbering scheme for NMR are shown in Figure 2.
protons and δ 3.80 ppm triplet of two protons, indicating that the protons corresponding to CH₂Cl-CH₂ moiety of BMH were half in number. In ¹³C NMR spectra, C-16 and C-17 were shift from δ 42.18 ppm and δ 46.59 ppm to δ 40.96 ppm and δ 42.18 ppm, respectively. ¹³C/DEPT NMR spectral data of impurity-A confirmed the presence of five methylene carbon atoms. This suggested that the BMH has two extra –CH₂ group δ 852.35 ppm at position C-16 and δ 840.96 ppm at position C-17 when compared with that of impurity-A. In COSY experiment, the upfield coupling correlation was found for –CH₂ group (H-11, δ 83.16 ppm; H-12, δ 82.40 ppm and H-13, δ 82.02 ppm). Another correlation was observed in the benzene ring (H-1, δ 87.71 ppm; H-2, δ 87.14 ppm and H-4, δ 87.07 ppm). The COSY and HMBC data revealed that the position C-16 of –CH₂ group and also C-17 of –CH₂ group. The HMBC spectrum showed correlations between C-14 (δ 173.64 ppm), H-13 (δ 82.40 ppm) and H-12 (δ 82.02 ppm). The four quaternary carbons of benzimidazole ring were assigned and the linkage based on HMBC correlations. According to the HSQC spectrum, the three tertiary carbons appearing at δ 61.15 ppm, 113.36 and 96.39 ppm were assigned to 2, 1 and 4 positions and five secondary carbons appearing at δ 52.35 ppm, δ 32.65 ppm, δ 24.09 ppm, δ 30.95 ppm and δ 112.43 ppm, respectively. In ¹³C NMR, all these carbon atoms were exchangeable with D₂O, indicating that impurity-B has one more active proton than BMH.

Based on the spectral data, the structure of impurity-A was characterized as 4-{5-(2-chloro-ethylamino)-1-methyl-1H-benzimidazol-2-yl} butyric acid hydrochloride. Comparative ¹H NMR and ¹³C NMR spectral data for BMH and impurity-A are given in Table I. The complete interpretation is tabulated for more clarity in Tables I and II.

**Structural elucidation of impurity-B**

The molecular ion peak of m/z 679 [M+H]+ by LC–MS analysis indicated a mass of 678 in molecular form which is 321 amu more than that of bendamustine. The high-resolution mass spectrum by Q-TOF/MS of impurity-B showed molecular ion peak at 679.2335 [M+H]+ in positive ion mode and 677.2178 [M–H]− in negative ion mode, indicating the elemental composition of impurity-B in molecular form is C₃₂H₄₁N₆O₄Cl₃. Under the long-term stability test of BMH, we found that the impurity-B was increased gradually while impurity-C was decreased gradually. This suggests that BMH and impurity-C may lose H₂O to form impurity-B.

One-dimensional ¹H NMR and ¹³C NMR analyses reveal that the impurity-B has two unique methyl groups (δ 83.90 ppm, s, 6H in ¹H NMR; δ 83.95 ppm and δ 83.91 ppm in ¹³C NMR) while BMH has only one methyl group. In ¹H NMR spectrum, the proton signals appearing at δ 81.5 ppm (2H, s, br) and δ 88.0 ppm (1H, s, br) were exchangeable with D₂O, indicating that impurity-B has one more active proton than BMH. Furthermore, a significant chemical shift was observed for the positions C-20, C-1, C-2, C-21, C-4 and C-23 from δ 87.73 ppm doublet of one proton, δ 87.72 ppm doublet of one proton, δ 87.12 ppm doublet of two protons, δ 86.94 ppm doublet of one proton and δ 86.93 ppm doublet of one proton and thus impurity-B has one more benzimidazole ring than BMH. In COSY experiment, the upfield coupling correlation was found for –CH₂ group (H-19; δ 84.19 ppm, H-18; δ 83.71 ppm). The protons appearing at δ 83.81 ppm (4H, t), δ 83.77 ppm (6H, m) and δ 83.76 ppm (2H, m) were assigned to position (35/35), (16/36/36) and 17, respectively. According to COSY and HMBC data, the protons at δ 30.95 ppm (4H, t), δ 82.02 ppm (2H, t), δ 82.04 ppm (2H, m) and δ 82.03 ppm (2H, m) are assigned to position (11/30), 32, 13, 31 and 12. ¹³C/DEPT NMR spectral data of impurity-B showed the presence of two primary carbons, 14 secondary carbons, six tertiary carbons and 10 quaternary carbons. All the quaternary carbons and secondary carbons are assigned and the linkage based on the HMBC correlations. According to the HSQC and HMBC spectrum, the six tertiary carbons appearing at δ 8113.40 ppm, δ 8113.25 ppm, δ 8112.43 ppm, δ 8112.34 ppm, δ 894.65 ppm and δ 894.56 ppm are assigned to 20, 1, 21, 2, 23 and 4 positions, respectively.
Based on the spectral data the structure of impurity-B was characterized as 4-[5-[[2-(4-[[bis-(2-chloroethyl)-amino]-1-methyl-1H-benzoimidazol-2-yl]-butyryloxy]-ethyl]-(2-chloroethyl)-amino]-1-methyl-3a,7a-dihydro-1H-benzoimidazol-2-yl] butyric acid hydrochloride. Comparison of 1H NMR and 13C NMR spectral data for BMH and impurity-B are given in Table I. The complete interpretation is tabulated for more clarity in Tables I and II.

Discussion

Under the stressed conditions, two degradant impurities in bendamustine hydrochloride drug product were detected by HPLC. These two impurities were isolated from preparative liquid chromatography, and were further characterized using Q-TOF/MS and NMR. The proposed formation of impurities is shown in Figure 3. Impurity-C was formed from BMH by hydrolysis reaction while impurity-A was formed from BMH by degradation reaction under the photostability testing. Further esterification reaction of impurity-C with BMH under the thermal stressed testing leads to the formation of impurity-B. Storage in dark at room temperature is important for ensuring the quality and safety of BMH drug product. We conducted the long-term stability test by keeping BMH drug product in dark condition at 25 °C with 60% relative humidity and impurity-B increased from 0.06% to 0.27% while impurity-A had no significant change in the 24th month.

Conclusion

Two degradant impurities have been identified in BMH drug product. These two impurities were isolated, identified and characterized using HPLC (analytical and preparative), MS, 1H NMR, 13C NMR and 2D NMR (HSQC, HMBC and COSY). It should be kept in mind that storage in dark at room temperature is important for ensuring the quality and safety of BMH drug product.

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