Metabolite Profiling of Bendamustine in Urine of Cancer Patients after Administration of [14C]Bendamustine

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ABSTRACT:

Bendamustine is an alkylating agent consisting of a mechlorethamine derivative, a benzimidazole group, and a butyric acid substituent. A human mass balance study showed that bendamustine is extensively metabolized and subsequently excreted in urine. However, limited information is available on the metabolite profile of bendamustine in human urine. The objective of this study was to elucidate the metabolic pathways of bendamustine in humans by identification of its metabolites excreted in urine. Human urine samples were collected up to 168 h after an intravenous infusion of 120 mg/m2 (80–95 μCi) [14C]bendamustine. Metabolites of [14C]bendamustine were identified using liquid chromatography-high-resolution-tandem mass spectrometry with off-line radioactivity detection. Bendamustine and a total of 25 bendamustine-related compounds were detected. Observed metabolic conversions at the benzimidazole and butyric acid moiety were N-demethylation and γ-hydroxylation. In addition, various other combinations of these conversions with modifications at the mechlorethamine moiety were observed, including hydrolysis (the primary metabolic pathway), cysteine conjugation, and subsequent biotransformation to mercapturic acid and thiol derivatives, N-dealkylation, oxidation, and conjugation with phosphate, creatinine, and uric acid. Bendamustine-derived products containing phosphate, creatinine, and uric acid conjugates were also detected in control urine incubated with bendamustine. Metabolites that were excreted up to 168 h after the infusion included products of N-demethylation and cysteine conjugation of bendamustine and γ-hydroxybendamustine. The range of metabolic reactions is generally consistent with those reported for rat urine and bile, suggesting that the overall processes involved in metabolic elimination are qualitatively the same in rats and humans.

Introduction

Bendamustine is an alkylating agent that is used for the treatment of chronic lymphocytic leukemia and indolent B-cell non-Hodgkin’s lymphoma that has progressed during or after treatment with a rituximab-containing regimen. It consists of a bifunctional mechlorethamine derivative, a benzimidazole heterocyclic ring, and a butyric acid moiety (Table 1).

Clinical and preclinical studies showed that bendamustine is extensively metabolized in vivo (Teichert et al., 2005, 2009; Chovan et al., 2007). Two phase 1 metabolites resulting from metabolic conversions at the benzimidazole/butyric acid moiety: γ-hydroxybendamustine and N-desethylbendamustine have been identified (Teichert et al., 2007). All other metabolic conversions occurred at the mechlorethamine moiety. In human bile, cysteine S-conjugates, mercapturic acid, and mercapturic acid sulfoxide conjugates were detected, suggesting an important role of the glutathione conjugation pathway in the metabolism of bendamustine, although intact glutathione conjugates were not observed (Teichert et al., 2009). In contrast, intact glutathione conjugates were observed in rat urine. Among the metabolites detected in rat urine and bile, three metabolites were identified as glutathione conjugates and six as mercapturic acid conjugates. In addition, products of N-dealkylation, oxidation, carboxylic acid formation, and sulfate conjugation were postulated (Chovan et al., 2007). Human urine has been investigated for the presence of cysteine S-conjugates (Teichert et al., 2005), but the presence of other metabolites and the excretion of bendamustine metabolites over time have not been described.

The objective of this study was to elucidate the metabolic pathways of bendamustine in humans by identification of the chemical structures of its metabolites excreted in urine. In a mass balance study with [14C]bendamustine in humans, 76% of the administered radiochemical dose was recovered in excreta collected in a period of up to 3...
weeks after administration of a single 60-min intravenous dose of 120 mg/m² (80–95 μCi) [14C]bendamustine hydrochloride (Dubbelman et al., 2011). Approximately one-half of the radiochemical dose was recovered in urine and approximately one-quarter in feces. Unchanged bendamustine in urine comprised only ~3% of the dose, indicating a major role of metabolism in the elimination of bendamustine. During the mass balance study, separate urine aliquots were prepared for metabolite profiling. From these aliquots, high concentration samples were selected to detect metabolites using a selective high-performance liquid chromatography (HPLC) method, followed by off-line radioactivity detection and characterization with a linear ion trap mass spectrometer. With use of existing knowledge of bendamustine metabolism and high-resolution mass spectrometry (MS), the metabolites were tentatively identified. In addition, their presence in urine was investigated over time, up to 168 h after the [14C]bendamustine infusion.

Materials and Methods

Reference Standards and Chemicals. Reference standards of bendamustine (4-[5-[bis[2-chloroethyl]amino]-1-methyl-benzoimidazol-2-yl]butyric acid hydrochloride), N-desmethylbendamustine (M4), and the product of monohydrolysis (HP1) were synthesized by Carbogen AMCIS AG (Bubendorf, Switzerland). Cephalon Inc. (Frazer, PA) manufactured γ-hydroxybendamustine (M3), and Salmedix Inc. (San Diego, CA) manufactured the product of dihydrolysis (HP2). Cephalon Inc. kindly provided all reference compounds. An overview of their structures is provided in Table 1.

Chemical structure of bendamustine and its metabolites for which reference standards were available

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
</tr>
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<tbody>
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<td>Bendamustine</td>
<td>Cl</td>
<td>Cl</td>
<td>CH3</td>
<td>H</td>
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<tr>
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<td>Cl</td>
<td>CH3</td>
<td>OH</td>
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<tr>
<td>N-Desmethylbendamustine (M4)</td>
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<td>Cl</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Product of monohydrolysis (HP1)</td>
<td>Cl</td>
<td>OH</td>
<td>CH3</td>
<td>H</td>
</tr>
<tr>
<td>Product of dihydrolysis (HP2)</td>
<td>OH</td>
<td>OH</td>
<td>CH3</td>
<td>H</td>
</tr>
</tbody>
</table>

The asterisk indicates the position of the 14C label. The letters a1, a2, b1, e indicate characteristic fragmentation points of bendamustine metabolites during MSn and refer to Table 2.

Radiochromatograms were constructed by plotting the radioactivity (after background subtraction) against the retention time. Fractions containing at least 1% of the total radioactivity in a chromatogram in one or more urine samples were selected for further characterization. At the retention time of these peaks, the mass spectra of predose and postdose samples were compared to find ions that were present in the postdose samples and absent in the predose samples. These molecular ion masses were added to the parent list, which was used for the data-dependent analysis of all following LC-MS measurements.

Metabolite Identification. Metabolite identification was performed by analysis of the LC-MS² data. Several tools were used to facilitate the identification:

- Investigation of isotope pattern in the full MS spectrum for the presence of one or two chlorine atoms. The presence of one chlorine atom was recognized by the typical isotope pattern of
two ions 2 Da apart (from the $^{35}\text{Cl}$ and $^{37}\text{Cl}$ isotopes) with a proportion of 3:1. The presence of two chlorine atoms was recognized by the typical isotope pattern consisting of three ions 2 Da apart with a proportion of 9:6:1.

• Comparison of detected molecular masses with masses in a list of possible metabolites. With use of literature on metabolites of bendamustine (Chovan et al., 2007; Teichert et al., 2009), a list was made with the $[M + H]^+$ values of potential combinations of bendamustine, $\gamma$-hydroxybendamustine, and N-desmethylbendamustine with reactions at the mechloretamine moiety.

• Retention times and fragmentation patterns of potential metabolites were compared with those of the available reference standards (Table 1; Fig. 1). To obtain the $\gamma$-hydroxy (M3) and N-desmethyl (M4) equivalents of monohydrolyzed bendamustine (HP1) and dihydrolyzed bendamustine (HP2), reference standards of M3 and M4 were incubated for 1 h at 60°C in water.

Fig. 1. High-resolution MS$^2$ spectra and proposed fragmentation of the reference standards of bendamustine, $\gamma$-hydroxybendamustine, and N-desmethylbendamustine.
The elemental composition of the proposed structures was confirmed by the analysis of high-resolution MS and MS² data. In addition, the high-resolution data were used to propose an elemental composition and, if possible, a structure for metabolites that were not identified with the normal-resolution data.

High-resolution MS was performed on selected samples using an LTQ Orbitrap XL, preceded by a Finnigan Surveyor MS Pump Plus (Thermo Fisher Scientific). The 180-min chromatographic method and instrumental settings were identical to those described under Metabolite Detection, except for the normalized collision energy, which was set at 40%, and the data-dependent acquisition settings, which were adapted to collect high-resolution MS² data of the most intense and the second most intense ion of the parent mass list. The resolution was set at 60,000.

Time Course. To determine the bendamustine metabolite profile over time, urine samples of each patient collected at 2, 6, 24, 48, 96, and 168 h after the start of the [14C]bendamustine infusion were analyzed using a 60-min LC-LSC-MS² method. The instrumental settings were identical to those described for the 180-min LC-LSC-MS² runs, except for the gradient composition. The gradient started with 95% of 0.1% formic acid in water and 5% of acetonitrile for 5 min. The acetonitrile percentage was increased to reach 10% at 15 min, 20% at 25 min, 40% at 35 min, 60% at 45 min, and 80% at 50 min. This composition was maintained for 4.9 min, followed by a return to the initial condition of 5% acetonitrile, which was held for 5 min.

Intravesical Formation of Metabolites. Because a previous study showed that the stability of bendamustine in urine is limited (Dubbelman et al., 2012), an additional experiment was performed to test for potential intravesical conversion of bendamustine. Hereeto, a control urine sample was spiked with bendamustine to a final concentration of 25 μg/ml and analyzed immediately (time 0) and after incubation for 1 h at 37°C, whereby the 60-min chromatographic method was applied without fraction collection. The presence of metabolites was qualitatively assessed in both aliquots by LC-tandem MS (LC-MS/MS).

Results

Metabolite Detection and Identification. The radiochromatograms of the 180-min LC-LSC-MS² runs revealed 25 peaks that each represented more than 1% of the total radioactivity in the sample of one or more patients. These peaks were bendamustine (26) and bendamustine-related compounds, assigned Met2 to Met25. Figure 2 shows a representative radiochromatogram of a 2-h urine sample. Radiochromatograms of the 2-h urine samples of all six patients are provided in Supplemental Fig. 1. One additional early-eluting peak (Met1) was detected in the 60-min radiochromatograms of urine samples collected at later time points (Fig. 3). For each compound, the retention time with both the 180- and 60-min HPLC methods, the proposed elemental composition, theoretical and observed [M + H]+ value, the major or characteristic product ions with their proposed origin, and proposed identification are summarized in Table 2. The proposed chemical structures can be found in Table 3.

Met1 was not prominently present (i.e., >1% of the total radioactivity) in any of the 2-h urine samples; however, this metabolite became prevalent over time (Fig. 3). In the 60-min HPLC run, the retention time was between 3 and 5 min. A molecular ion was not identified by LC-MS analysis, and, therefore, the structure of Met1 was not determined.

The molecular ion of Met2 was detected at m/z 418 and comparison with the list of potential metabolites suggested a sulfate conjugate of didechlorinated γ-hydroxybendamustine. However, this was not confirmed by the high-resolution data. The high-resolution m/z of 418.1363 suggested an elemental composition of C₁₆H₂₄N₃O₈P. Major product ions were 338.1075 (−79.9658 Da, loss of HPO₄), 320.1600 (−97.9763 Da, loss of H₂PO₄), and 302.1495 (−115.9868 Da, loss of H₃PO₄ and water). The MS² spectrum of the product ion at m/z 338 (Fig. 3) was identical to the MS² spectrum of the dihydrolysis product of γ-hydroxybendamustine obtained by degradation of M3. Met2 was therefore proposed to be a phosphate conjugate of didechlorinated γ-hydroxybendamustine.

Met3 had a retention time of 10.9 and 18.7 min on the 60- and 180-min HPLC runs, respectively. The retention times and the MS² spectrum were identical to those of the product of dihydrolysis of N-desmethyl-bendamustine obtained by degradation of M4, which was therefore the proposed identity of Met3. This was confirmed with the high-resolution data.

Met4, with a protonated ion at m/z 441, was tentatively identified as a cysteine conjugate of didechlorinated γ-hydroxybendamustine on the basis of the list of potential metabolites. This proposed structure was supported by the high-resolution m/z value and by the product ions at m/z 352.1320 (−89.047 Da, loss of alanine) and m/z 294.1444 (−147.0346 Da, loss of ethylcysteine).

The ion of Met5 at m/z 402 suggested a sulfate conjugate of didechlorinated bendamustine. However, the high-resolution mass pointed to an elemental composition of C₁₀H₁₂N₃O₃P. The same
neutral losses were observed as for Met2: at $m/z$ 322.1756 (−79.9659 Da, loss of HPO₃), at $m/z$ 304.1650 (−97.9765 Da, loss of H₃PO₄), and at $m/z$ 286.1545 (−115.9870 Da, loss of H₃PO₄ and water). Because the MS³ spectrum of the product ion at $m/z$ 322 showed the same fragmentation pattern as the MS² spectrum of the reference standard of dihydrolyzed bendamustine, the proposed structure of Met5 was a phosphate conjugate of didechlorinated bendamustine.

Metabolite Met6 had a retention time of 13.3 and 23.8 min on the 60- and 180-min HPLC runs, respectively. The retention times, MSⁿ spectra, and high-resolution data were identical to those of the product of dihydrolysis of γ-hydroxybendamustine (obtained by thermal degradation of M3), which was therefore the proposed identity of Met6.

The protonated ion of Met7, found at $m/z$ 417 (417.2234 with high-resolution MS), was not present in the list of potential metabolites. The MSⁿ spectra, and high-resolution data were identical to those of the product of dihydrolysis of γ-hydroxybendamustine (obtained by thermal degradation of M3), which was therefore the proposed identity of Met6.

The protonated ion of Met8, found at $m/z$ 417 (417.2234 with high-resolution MS), was not present in the list of potential metabolites. The most abundant product ion at $m/z$ 304.1650 (−97.9765 Da, loss of H₃PO₄), at $m/z$ 286.1545 (−115.9870 Da, loss of H₃PO₄ and water). Because the MS³ spectrum of the product ion at $m/z$ 322 showed the same fragmentation pattern as the MS² spectrum of the reference standard of dihydrolyzed bendamustine, the proposed structure of Met5 was a phosphate conjugate of didechlorinated bendamustine.

Metabolite Met6 had a retention time of 13.3 and 23.8 min on the 60- and 180-min HPLC runs, respectively. The retention times, MSⁿ spectra, and high-resolution data were identical to those of the product of dihydrolysis of γ-hydroxybendamustine (obtained by thermal degradation of M3), which was therefore the proposed identity of Met6.

The protonated ion of Met7, found at $m/z$ 417 (417.2234 with high-resolution MS), was not present in the list of potential metabolites. The MSⁿ spectra, and high-resolution data were identical to those of the product of dihydrolysis of γ-hydroxybendamustine (obtained by thermal degradation of M3), which was therefore the proposed identity of Met6.

The protonated ion of Met8, found at $m/z$ 417 (417.2234 with high-resolution MS), was not present in the list of potential metabolites. The most abundant product ion at $m/z$ 304.1650 (−97.9765 Da, loss of H₃PO₄), at $m/z$ 286.1545 (−115.9870 Da, loss of H₃PO₄ and water). Because the MS³ spectrum of the product ion at $m/z$ 322 showed the same fragmentation pattern as the MS² spectrum of the reference standard of dihydrolyzed bendamustine, the proposed structure of Met5 was a phosphate conjugate of didechlorinated bendamustine.

The MSⁿ value of the protonated ion of Met9 (472.1923 with high-resolution MS), was not present in the list of potential metabolites. The presence of product ions at $m/z$ 304.1651 and 278.1494, which were also detected in the MS² spectrum of HP2, and the absence of a 37Cl isotope suggested that Met11 was a 168.0272-Da conjugate of didechlorinated bendamustine. The proposed elemental composition of the conjugate was C₅H₄N₄O₃ (168.0273 Da; Δppm = 0.57), which corresponds with the endogenous compound uric acid. Neutral loss of 43 Da (HCON), followed by loss of 28 Da (CO), is frequently observed for uric acid derivatives (Bianco et al., 2009) and also occurred for Met11, supporting the proposed identification of this compound as a uric acid conjugate of didechlorinated bendamustine.

The protonated ion of Met12, detected at $m/z$ 454 (454.1805 with high-resolution MS), was also not present in the list of potential metabolites and, similar to Met11, the presence of the HP2-related product ions at $m/z$
### TABLE 2
Overview of structural information obtained by LC-MSn for bendamustine metabolites detected in human urine

<table>
<thead>
<tr>
<th>Peak Identification</th>
<th>$T_{a}^{a}$</th>
<th>$T_{b}^{b}$</th>
<th>Proposed Elemental Composition</th>
<th>Theoretical</th>
<th>Observed</th>
<th>Δppm</th>
<th>Major or Specific Product Ions</th>
<th>Proposed Metabolic Conversions at:</th>
<th>Chloroethyl Moiety 1</th>
<th>Chloroethyl Moiety 2</th>
<th>Benzimidazole/Butyric Acid Moiety</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>−2.63</td>
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<td>Unknown Phosphate conjugation</td>
<td>Unknown γ-Hydroxylation</td>
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</tr>
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<td>2</td>
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<td>308.1605</td>
<td>308.16</td>
<td>−1.62</td>
<td>263.126 (e1), 214.0971 (e1 + b2 + f)</td>
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<td>Hydrolysis</td>
<td>N-Demethylation</td>
<td></td>
</tr>
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<td>441.179</td>
<td>−2.72</td>
<td>352.1320 (h), 294.1444 (e1), 276.1338 (e1 + f)</td>
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<td>γ-Hydroxylation</td>
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<td>γ-Hydroxylation</td>
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<td>10 (HP2)</td>
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<td>472.1923</td>
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<td>Uric acid conjugation</td>
<td>Hydrolysis</td>
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<td>435.1894</td>
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<td>338.1525</td>
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<td>424.1624 (h), 354.1572 (m), 336.1468 (m + f), 322.1311 (a1), 304.1206 (a1 + f)</td>
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<td>320.1155 (2f), 294.1363 (e)</td>
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<td>γ-Hydroxylation</td>
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<td>18</td>
<td>72.5</td>
<td>28.1</td>
<td>C$<em>{18}$H$</em>{26}$N$<em>{2}$O$</em>{4}$</td>
<td>296.1160</td>
<td>296.1153</td>
<td>−2.36</td>
<td>278.1049 (f), 250.1100 (e), 242.1283 (a2 + f)</td>
<td>N-Dealkylation</td>
<td>Hydrolysis</td>
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<td></td>
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<tr>
<td>19</td>
<td>73.7</td>
<td>28.5</td>
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<td>443.1514</td>
<td>443.1503</td>
<td>−2.48</td>
<td>354.1034 (h), 296.1157 (e1), 278.1052 (e1 + f)</td>
<td>Cysteine conjugation</td>
<td>Hydrolysis</td>
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<td>29.2</td>
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<td>340.1416</td>
<td>−1.76</td>
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<tr>
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<td>485.1605</td>
<td>−3.09</td>
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<td>356.119</td>
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<td>Thiol conjugation</td>
<td>Hydrolysis</td>
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<tr>
<td>23</td>
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<td>35.1</td>
<td>C$<em>{18}$H$</em>{26}$N$<em>{2}$O$</em>{4}$</td>
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<td>374.1023</td>
<td>−2.67</td>
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<td>Hydrolysis</td>
<td>Hydrolysis</td>
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</table>
304.1651 and 278.1495 and the absence of a $^{37}$Cl isotope suggested that Met12 was a 150.0154-Da conjugate of dichlorinated bendamustine. However, the structure of the conjugate was not identified.

The protonated ion of Met13, found at m/z 435 (435.1894 with high-resolution MS), was not in the list of potential metabolites. The isotope pattern indicated the presence of one chlorine atom. The most abundant product ion was m/z 322.1311, which was equal to the fragment ion of bendamustine originating from loss of HCl. The corresponding neutral loss (−113.0583 Da) was identical to the neutral loss observed for Met7; therefore, the proposed identity of Met13 is a creatinine conjugate of bendamustine.

The m/z value of the protonated ion of Met14 (m/z 312) corresponded with N-dealkylated γ-hydroxybendamustine in the list of potential metabolites. This identification was supported by the specific isotope pattern of one chlorine atom and by the high-resolution m/z value.

The similarity of the MS$^2$ spectrum of Met15 (protonated ion at m/z 338) with the MS$^2$ spectrum of Met8 suggested that Met15 was a part of Met8, and the proposed identity was a thiol conjugate of dichlorinated bendamustine. This was supported by the absence of a $^{37}$Cl isotope and by the high-resolution m/z value.

The protonated ion of Met16 at m/z 420 suggested a sulfate conjugate of bendamustine. The MS$^3$ spectrum of the product ion at m/z 340 was identical to the MS$^3$ spectrum of monodechlorinated bendamustine (Fig. 3). However, in line with Met5 and Met2, the high-resolution mass and the neutral losses of the most abundant product ions at m/z 340.1418 (−79.9658 Da, loss of HPO$_4^−$), 322.1313 (−97.9763 Da, loss of H$_2$PO$_4^−$), and 304.1207 (−115.9869 Da, loss of H$_2$PO$_4$ and water) pointed to a phosphate conjugate of bendamustine.

Met17 had a retention time of 25.9 and 71.1 min with the 60- and 180-min HPLC methods, respectively. The protonated ion, detected at m/z 483 (483.2353 with high-resolution MS), was not in the list of potential metabolites. The presence of a product ion at m/z 322.1311, which was also detected in the MS$^2$ spectrum of HP1, and the isotope pattern specific for one chlorine atom suggested that Met17 was a monodechlorinated 161.1042-Da conjugate of bendamustine. However, a complete structure was not determined.

Met18 had a retention time of 27.1 and 71.3 min with the 60- and 180-min HPLC methods, respectively. The retention times, MS$^n$ spectra, and high-resolution data were identical to those of the product of monohydration of γ-hydroxybendamustine, which was therefore the proposed identity of Met18.

The m/z value of the protonated ion of Met19 (m/z 296) corresponded with N-dealkylated bendamustine in the list of potential metabolites. The high-resolution m/z value supported this identification. The m/z value of the protonated ion of Met20 (m/z 443) corresponded with a cysteine conjugate of bendamustine in the potential metabolites list. This identification was supported by the high-resolution m/z value and by the neutral losses that were similar to Met4 and Met8: at m/z 354.1034 (−89.0469 Da, loss of alanine) and at m/z 296.1157 (−147.0347 Da, loss of ethylcysteine).

The protonated ion of Met21, detected at 29.2 and 82.7 min with the 60- and 180-min HPLC methods, respectively. Because the retention times, MS$^n$ spectra, and high-resolution data of this metabolite were equal to those of the reference standard of HP1, Met21 was identified as monohydration of bendamustine (HP1).

The m/z value of the protonated ion of Met22 (m/z 485) corresponded with an N-acetylcycteine (mercapturic acid) conjugate of bendamustine in the list of potential metabolites. This identification was supported by the high-resolution m/z value and the neutral losses of the most abundant product ions at m/z 356.1187 (−129.0418 Da, loss of N-acetyl-2-iminopropionic acid) and at m/z 296.1155 (−189.045 Da, loss of N-acetyl-2-ethylcysteine).

The protonated ion of Met23, detected at m/z 356, was not included in the list of potential metabolites. The similarity of the MS$^3$ spectrum of this metabolite with the MS$^3$ spectrum of Met22 suggested that Met23 was a part of Met22, and the proposed identity was a thiol conjugate of bendamustine, which was supported by the presence of one $^{37}$Cl isotope and by the high-resolution m/z value.

Based on comparison of the retention times, MS$^n$ fragmentation patterns, and high-resolution data with reference standards, the compounds designated Met24, Met25, and 26 were identified as γ-hydroxybendamustine (M3), N-desmethyldendamustine (M4), and bendamustine, respectively.

**Time Course.** The radiochromatograms showing the metabolite profile of bendamustine in urine over time from a representative patient are provided in Fig. 4. The designation of the peaks was based on the detection and retention times in LC-MS/MS chromatograms. Table 4 summarizes each metabolite and all analyzed time points in the patients in whom each metabolite was detected.

The metabolite profiles show a rapid conversion of bendamustine (26) into more polar metabolites. Low but detectable levels of five metabolites were present in most late urine samples, collected up to 168 h after the administration of the radiolabeled bendamustine. Apart from the unidentified metabolite (Met1), these were the dihydrolysis products of bendamustine (Met10) and γ-hydroxybendamustine (Met6) and the cysteine conjugates of dichlorinated bendamustine (Met8) and γ-hydroxybendamustine (Met4).
Intravesical Formation of Metabolites. Table 4 shows the metabolites formed after a 1-h incubation at 37°C of urine spiked with bendamustine. In addition to hydrolysis, which was known to occur in urine, conjugation with phosphate, creatinine, and uric acid also occurred.

Discussion

This study investigated the metabolite profile of bendamustine in urine of patients with relapsed or refractory malignancies to whom [14C]bendamustine had been administered via intravenous infusion. A total of 25 bendamustine-related compounds were detected in addition to the parent drug. Observed metabolic conversions at the benzimidazole and butyric acid moiety were N-demethylation and γ-hydroxylation. In addition, various combinations of these conversions with modifications at the mechlorethamine moiety were observed, including hydrolysis, cysteine conjugation, and subsequent biotransformation to mercapturic acid and thiol derivatives, N-dealkylation, oxidation, and conjugation with, among others, phosphate, creatinine, and uric acid. Most metabolites were predominantly present in early collections of excreta, except for five: one unidentified metabolite (Met1) and the products of dihydrolysis and of cysteine conjugation of bendamustine (Met10 and Met8) and γ-hydroxybendamustine (Met6 and Met4), which were excreted up to 168 h after the infusion. In addition, two other minor metabolic conversions were tentatively identified as products of conjugation with partially identified compounds (Met12 and Met17). Although bendamustine primarily activates base excision repair pathways (Leoni et al., 2008), repaired DNA adducts of bendamustine have not been identified in the urine samples.

Use of knowledge-based prediction of metabolites proved to be very helpful in this study. More than half of the 25 bendamustine-derived compounds were included in the list of potential metabolites, a list that was made beforehand, consisting of [M + H]+ values of potential combinations of known and theoretical metabolic conversions of bendamustine. On the basis of this list, however, three metabolites were initially thought to be sulfate conjugates. Sulfate conjugation was assumed in rat urine (Chovan et al., 2007), and, moreover, the normal-resolution MS² spectra of these metabolites showed intense product ions resulting from loss of 80 Da, which is characteristic for sulfate conjugates (Levens et al., 2005). However, evaluation of the elemental composition of the proposed metabolite structures with high-resolution MS revealed that the mass deviations
for the three postulated sulfate conjugates (approximately 20 ppm) were substantially higher than deviations of the other metabolites (range, −1 to −3 ppm). This led to their tentative identification as phosphate conjugates. This is an excellent example of the additional value of high-resolution MS above normal-resolution MS for metabolite identification.

The types of metabolites that were observed in this study were generally consistent with those reported previously for rat (Chovan et al., 2007). The most abundant metabolite in rat urine and bile was a mercapturic acid conjugate of bendamustine, corresponding with Met22 in this study. The similarities between the bendamustine metabolites found in rat and human suggest that the overall processes involved in metabolic elimination of bendamustine are qualitatively similar for the two species. However, the origin of the cysteine-related metabolites may be different. Glutathione conjugation was suggested to be a major detoxification pathway in rats, based on the observation of glutathione conjugates and possible biotransformation products thereof (mercapturic acid conjugates) in rat urine and bile. In human bile, glutathione conjugates were not observed, and this was explained by a higher hepatic activity of γ-glutamyltransferase in human compared with rat (Teichert et al., 2009). This study showed that cysteine-related metabolites continued to be excreted in urine collected up to 168 h after bendamustine administration. However, cysteine-related metabolites originating from glutathione conjugation would be expected to be excreted quickly. Therefore, although the early excreted cysteine conjugates may theoretically be derived from glutathione conjugation, another potential source of the long-lived cysteine conjugates may be their release during catabolism of alkylated proteins. In addition, the other long-lived metabolites, the dihydrolysis products of bendamustine and γ-hydroxybendamustine, may be derived from slow hydrolysis of alkylated products. A similar origin may apply to the highly polar metabolite Met1, the identity of which was not elucidated.

Metabolic products of bendamustine that were not observed in previous studies in rat and/or human were thiol derivatives; phos-
In conclusion, this study showed that bendamustine is extensively metabolized in humans via hydrolytic, oxidative, and conjugative pathways. These metabolic reactions are similar to those identified in rats. In addition, products were observed that are consistent with intravesical conversion of bendamustine. Analysis of time points at which metabolites were detected in patients also indicates that their conjugation in the control urine after incubation with bendamustine. This shows that the bendamustine-derived products of bendamustine were detected in control urine after incubation with bendamustine. This indicates that intravesical formation may be an explanation for their unexpected presence in the metabolite profile of bendamustine. Expected in the conjugate. However, the complete structure has not been elucidated. The high-resolution mass of the conjugate of Met17 (161.1046 Da) led to a proposed elemental composition of C_{10}H_{15}NO_{3} (161.1046 Da; Δppm = −2.48), a molecular formula corresponding with that of carnitine. The quaternary ammonium compound can be endogenously formed and is required for transport of long-chain fatty acids across intracellular membranes, which is facilitated by oxidation of the fatty acids into acylcarnitines (Strijbis et al., 2010). The suggestion of Met17 being a carnitine conjugate of bendamustine is supported by the most abundant product ion at m/z 242.1624, because the loss of 59.0729 Da (loss of trimethylamine) is characteristic for carnitine and acylcarnitine conjugates (Minkler et al., 2005). Although the mass spectra give no definite answer on the site of the conjugation, it is hypothesized that Met17 may be monohydrolyzed bendamustine, conjugated with carnitine at the butyric acid moiety to form an acylcarnitine derivative. This would be in line with the acylcarnitine conjugates that have been observed for other carboxylic acid-containing drugs (Millington et al., 1985; Olsen et al., 2005). The suggestion of Met17 being a carnitine conjugate of bendamustine is supported by the most abundant product ion at m/z 242.1624, because the loss of 59.0729 Da (loss of trimethylamine) is characteristic for carnitine and acylcarnitine conjugates (Minkler et al., 2005). Although the mass spectra give no definite answer on the site of the conjugation, it is hypothesized that Met17 may be monohydrolyzed bendamustine, conjugated with carnitine at the butyric acid moiety to form an acylcarnitine derivative. This would be in line with the acylcarnitine conjugates that have been observed for other carboxylic acid-containing drugs (Millington et al., 1985; Olsen et al., 2005).

In conclusion, this study showed that bendamustine is extensively metabolized in humans via hydrolytic, oxidative, and conjugative pathways. These metabolic reactions are similar to those identified in rats. In addition, products were observed that are consistent with intravesical reaction of bendamustine and certain of its metabolites with uric acid, creatinine, and phosphate.

### Authorship Contributions

**Participated in research design:** Dubbelman, Jansen, Rosing, Darwish, Hellriegel, Robertson, Schellens, and Beijnen.  
**Conducted experiments:** Dubbelman and Jansen.  
**Performed data analysis:** Dubbelman and Jansen.
Wrote or contributed to the writing of the manuscript: Dubbelman, Jansen, Rosing, Darwish, Hellriegel, Robertson, Schellens, and Beijnen.

References


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Correction to “Metabolite Profiling of Bendamustine in Urine of Cancer Patients after Administration of [14C]Bendamustine”

In the above article [Dubbelman AC, Jansen RS, Rosing H, Darwish M, Hellriegel E, Robertson P Jr, Schellens JHM, Beijnen JH (2012) Drug Metab Dispos 40:1297–1307, doi 10.1124/dmd.112.045229], an erroneous drawing of the core-structure of bendamustine was copied and used throughout the paper to present bendamustine metabolites. The erroneous drawing was presented for the structure of bendamustine and its metabolites in Table 1, Table 3, Figure 1 and Figure 3. A revised Table 1 is offered below.

The systematic name of bendamustine (4-[5-[bis(2-chloroethyl)amino]-1-methyl-benzoimidazol-2-yl]butyric acid, as was correctly stated in the Materials and Methods section) indicates that the bis(2-chloroethyl)amino group is situated at the 5-position of the benzimidazole moiety. However, due to a clerical error this group is drawn at the 6-position in the structure presented for bendamustine in Table 1 of the original article. The erroneous drawing of the core-structure of bendamustine was copied and used throughout the paper to present bendamustine metabolites. The correct structure of bendamustine is presented below in the revised Table 1. Likewise, in all molecular structures of Table 3, Figure 1 and Figure 3, the bis(2-chloroethyl)amino group or its derivative should be situated at the 5-position of the benzimidazole moiety. The proposed fragmentation pathways remain unchanged and the results and conclusions of the original article are by no means affected by this error.

The authors regret any inconvenience caused by this error.

TABLE 1
Chemical structure of bendamustine and its metabolites for which reference standards were available

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*The asterisk indicates the position of the 14C-label. The letters a1, a2, b1…e indicate characteristic fragmentation points of bendamustine metabolites during MSn and refer to Table 2.
Metabolite Profiling of Bendamustine in Urine of Cancer Patients after Administration of [14C]Bendamustine
Anne-Charlotte Dubbelman, Robert S. Jansen, Hilde Rosing, Mona Darwish, Edward Hellriegel, Philmore Robertson, Jr., Jan H.M. Schellens, and Jos H. Beijnen

Supplemental Figure 1 (part 1 of 2): Radiochromatograms of the urine samples of six cancer patients, collected 2 h after start of a 1-h infusion with [14C]-bendamustine (120 mg/m², ~95 µCi).
Supplemental Figure 1 (part 2 of 2): Radiochromatograms of the urine samples of six cancer patients, collected 2 h after start of a 1-h infusion with $^{14}$C-bendamustine (120 mg/m², ~95 µCi).