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 Ci) [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 dihydrolysis 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-desmethylbendamustine 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-desethylbendamustine (M4), and the product of monohydrolysis (HP1) were synthesized by Carbogen AMCIS AG (Bubendorf, Switzerland). Cephalon Inc. (Frazer, PA) manufactured γ-hydroxybendamustine (M3) and, 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](image)

#### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
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<td>CH3</td>
<td>H</td>
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<tr>
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<td>OH</td>
<td>OH</td>
<td>CH3</td>
<td>H</td>
</tr>
</tbody>
</table>

**Methanol (SupraGradient grade) and acetonitrile (SupraGradient grade) were obtained from Biosolve Ltd. (Valkenswaard, The Netherlands). Formic acid (≥98%) and water (LiChrosolv) were purchased from Merck (Darmstadt, Germany) and Ultima Gold liquid scintillation cocktail from PerkinElmer Life and Analytical Sciences (Waltham, MA).**

**Samples.** The urine samples used in this study were collected during a human mass balance study with [14C]bendamustine (Dubbelman et al., 2011). Each void was collected individually, and aliquots were stored at nominally −70°C. Upon analysis of a sample, one urine aliquot was thawed on ice water and divided over autosampler vials. One autosampler vial was used for immediate analysis and the others were refrozen for additional analyses.

All patients gave their informed consent before participation in the mass balance study and the study was conducted in accordance with the guidelines for Good Clinical Practice, the code of Federal Regulations title 21 (parts 50, 54, 56, 312, and 314), and the European Clinical Trials Directive (2001/20/EC).

**Metabolite Detection.** Urine samples collected 2 h after the start of the infusion of [14C]bendamustine (which was the urine portion with the highest radioactive concentration for five of six patients) were submitted to 180-min HPLC with off-line radiodetection by liquid scintillation counting (LSC) and mass spectrometric detection [liquid chromatography (LC)-LSC-MS²]. The predose urine samples were analyzed using this method as well; however, no radiodetection was performed.

Individual urine aliquots, stored at −70°C, were thawed, vortex-mixed, and analyzed immediately. An Accela autosampler (Thermo Fisher Scientific, Waltham, MA), thermostatted at 4°C, loaded sample volumes of 50 μl onto a Synergi Polar RP column (4.6 mm i.d. × 150 mm, 4-μm particle size; Phenomenex, Torrance, CA) preceded by an in-line filter (0.2 μm; Upchurch Scientific, Oak Harbor, WA). The HPLC pump (Accela; Thermo Fisher Scientific) maintained a flow rate of 1.0 ml/min and started a gradient elution with 100% of 0.1% formic acid in water, followed by a slow linear increase of organic solvent (acetonitrile) to reach 12.5% acetonitrile at 75 min, 50% at 150 min, and 80% at 180 min.

A postcollection accurate flow splitter (LC Packings, Sunnyvale, CA) directed one-fourth of the flow to an LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific) and three-fourths to a fraction collector (LKB-FRAC-100; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

During the first 4 min, the MS eluent was directed to waste using a switching valve. The mass spectrometer was equipped with an electrospray ionization probe and operated in the positive ion mode, with a spray voltage of 5.4 kV, a capillary temperature of 300°C, and a capillary voltage of 6.5 V. The sheath, auxiliary, and sweep gas flows were optimized to 60, 10, and 5 arbitrary units, respectively. Wideband activation was enabled, the scan range was 100 to 1100 atomic mass units, the isolation width was 2.0, and the normalized collision energy used for collision-induced dissociation was 35%. Data-dependent acquisition of MS² and MS³ spectra was based on a predefined parent list containing nLe values of known bendamustine metabolites.

The eluent directed to the fraction collector was collected in 6-ml plastic LSC vials at a rate of 1 min/vial. After the addition of 4 ml of quenched radioactive reference standards. A calibration curve of quenched radioactive reference standards was used to correct for quenching. Samples were counted to a sigma 2 error of 1% with a maximum of 20 min.

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 $[\text{M} + \text{H}]^+$ values of potential combinations of bendamustine, γ-hydroxybendamustine, and N-desmethylbendamustine with reactions at the mechlorethamine 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 γ-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, γ-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. Here, 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 C₁₆H₂₄N₃O₇P. Major product ions were 338.1075 (−79.9658 Da, loss of HPO₄), 320.1600 (−97.9763 Da, loss of H₂PO₄), and 302.1495 (−115.9686 Da, loss of H₂PO₄ and water). The MS³ 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 C₁₆H₂₄N₃O₇P. 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 H₂PO₄ 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 most abundant product ion at m/z 304.1651 (–113.0583 Da) fragmented identically to the fragment at m/z 304 in the MS² spectrum of dihydrolyzed bendamustine, suggesting that Met7 was a 113.0583-Da conjugate of didechlorinated bendamustine. The proposed elemental composition of the conjugate was C₆H₇N₃O (113.0584 Da; Δppm = –0.88), which corresponds with the endogenous compound creatinine. Met7 was therefore proposed to be a creatinine conjugate of didechlorinated bendamustine.

The m/z value of the protonated ion of Met8 (m/z 425) corresponded with a cysteine conjugate of didechlorinated bendamustine in the list of potential metabolites. This identification was supported by the high-resolution m/z value and by the neutral losses that were similar to Met4: at m/z 336.1371 (–89.047 Da, loss of alanine) and at m/z 278.1494 (–147.0347 Da, loss of ethylcysteine).

The m/z value of the protonated ion of Met9 (m/z 276) corresponded with oxidized N-dealkylated bendamustine in the list of potential metabolites. The identification was supported by the high-resolution m/z value and by the major product ions 258.1232 (–18.0104 Da, loss of water), 230.1283 (–46.0053 Da, loss of carboxyl moiety), and 217.0972 (–59.0364, loss of aminoacetaldehyde), which are consistent with those reported for this compound in rat urine (Chovan et al., 2007).

Met10 eluted at 17.6 and 33.7 min with the 60- and 180-min HPLC methods, respectively. Based on comparison of the retention times, MSⁿ spectra, and the high-resolution data of Met10 with the available reference standards, Met10 was identified as dihydrolyzed bendamustine (HP2).

The protonated ion of Met11, found at m/z 472 (472.1923 with high-resolution MS), was not present in the list of potential metabolites. Based on comparison of the retention times, MSⁿ spectra, and the high-resolution data of Met10 with the available reference standards, Met10 was identified as dihydrolyzed bendamustine (HP2).

The protonated ion of Met11, found at m/z 472 (472.1923 with high-resolution MS), was not present in the list of potential metabolites. Based on comparison of the retention times, MSⁿ spectra, and the high-resolution data of Met10 with the available reference standards, Met10 was identified as dihydrolyzed bendamustine (HP2).
| Peak Identification | $T_{R}$ (min) | $\Delta T_{R}$ (s) | Proposed Elemental Composition | Theoretical $m/z$ | Observed $m/z$ | $\Delta m$ (ppm) | Major or Specific Product Ions [M + H]$^+$ | Proposed Metabolic Conversions at: | Chloroethyl Moiety 1 | Chloroethyl Moiety 2 | Benzimidazole/Butyric Acid Moiety |
|---------------------|----------------|-------------------|-------------------------------|------------------|----------------|----------------|------------------------------------------|-------------------------------|-------------------|-------------------|
| 1                   | 3-5             | 7.9               | Unknown                       | 418.1374         | 418.1363       | -0.23          | 338.1705 (g), 320.1600 (g + f), 302.1495 (g + 2f) | Unknown Phosphate conjugation | Unknown Hydrolysis | Unknown γ-Hydroxylation |
| 2                   | 14.7            | 7.9               | C$_{16}$H$_{24}$N$_{10}$O$_{6}$P | 308.1605         | 308.16         | -1.62          | 263.1260 (e1), 214.0971 (e1) | Hydrolysis | Hydrolysis | N-Demethylation |
| 3                   | 18.7            | 10.9              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$S | 441.1802         | 441.179        | -2.72          | 352.1320 (h), 294.1444 (e1), 276.1338 (e1) | Cysteine conjugation | Hydrolysis | γ-Hydroxylation |
| 4                   | 19.7            | 11.0              | C$_{16}$H$_{22}$N$_{10}$O$_{6}$S | 402.1425         | 402.1415       | -0.29          | 322.1756 (g), 304.1650 (h) | Phosphate conjugation | Hydrolysis | |
| 5                   | 22.0            | 12.1              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$P | 338.1710         | 338.1704       | -1.77          | 304.1496 (df), 276.1703 (e + f) | Hydrolysis | Hydrolysis | γ-Hydroxylation |
| 6                   | 23.8            | 13.3              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 427.2245         | 417.2234       | -2.64          | 304.1651 (a1), 278.1495 (c1) | Creatine conjugation | Hydrolysis | |
| 7                   | 24.2            | 13.9              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$S | 425.1853         | 425.1841       | -0.82          | 336.1371 (h), 276.1494 (c1) | Cysteine conjugation | Hydrolysis | |
| 8                   | 29.0            | 15.8              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$S | 276.1343         | 276.1336       | -2.51          | 304.1653 (f), 277.1418 (e1), 264.1234 (c1 + b2) | Hydrolysis | Hydrolysis | |
| 9                   | 29.4            | 16.9              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 332.1761         | 332.1754       | -2.17          | 429.1871 (l), 411.1766 (i + f) | Hydrolysis | Uric acid conjugation | Hydrolysis |
| 10 (HP2)            | 33.7            | 17.6              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 472.1939         | 472.1923       | -3.39          | 401.1650 (a1), 286.1545 (a1 + f) | Conjugation with 150.0154 Da | Hydrolysis | |
| 11                  | 40.7            | 18.9              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 454.1820         | 454.1805       | -3.30          | 408.1757 (k), 304.1651 (a1) | Conjugation with 150.0154 Da | Hydrolysis | |
| 12                  | 50.2            | 19.9              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 308.1605         | 308.16         | -1.62          | 263.1260 (e1), 214.0971 (e1) | Hydrolysis | Hydrolysis | N-Demethylation |
| 13                  | 54.6            | 23.1              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 435.1906         | 435.1894       | -0.76          | 304.1651 (a1), 296.1155 (c1) | Creatine conjugation | Hydrolysis | |
| 14                  | 57.3            | 25.4              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 312.1109         | 312.1103       | -1.92          | 276.0894 (2f), 248.0945 (g + f) | N-Dealkylation | Hydrolysis | γ-Hydroxylation |
| 15                  | 64.4            | 24.2              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 338.1533         | 338.1525       | -2.23          | 278.1494 (e1), 260.1388 | Thioli conjugation | Hydrolysis | |
| 16                  | 70.7            | 26.1              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 420.1086         | 420.1076       | -2.38          | 304.1651 (a1) | Conjugation with 161.1042 Da | Hydrolysis | Hydrolysis | γ-Hydroxylation |
| 17                  | 71.1            | 25.9              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 483.2363         | 483.2353       | -2.07          | 424.1624 (i), 354.1572 (m), 336.1468 | Hydrolysis | Conjugation with 161.1042 Da | Hydrolysis | γ-Hydroxylation |
| 18                  | 71.3            | 27.1              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 356.1372         | 356.1362       | -2.81          | 320.1155 (2f), 296.1363 | Hydrolysis | Hydrolysis | γ-Hydroxylation |
| 19                  | 72.5            | 28.6              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 296.1160         | 296.1153       | -2.36          | 278.1049 (f), 250.1100 (e) | N-Dealkylation | Hydrolysis | |
| 20                  | 73.7            | 26.5              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 443.1514         | 443.1503       | -2.38          | 354.1034 (b), 296.1157 (c1) | Cysteine conjugation | Hydrolysis | |
| 21 (HP1)            | 82.7            | 29.2              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 340.1422         | 340.1416       | -1.76          | 322.1314 (f), 286.1547 (a + b2) | Hydrolysis | Hydrolysis | |
| 22                  | 97.6            | 32.2              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 485.1620         | 485.1605       | -0.93          | 356.1187 (a1), 296.1155 (c1) | N-Acetylcysteine conjugation | Thioli conjugation | Hydrolysis |
| 23                  | 99.4            | 35.1              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 356.1194         | 356.119        | -1.12          | 302.1320 (a + b, 2f), 296.1160 (c1) | Thioli conjugation | Hydrolysis | |
| 24 (M3)             | 104.5           | 35.1              | C$_{16}$H$_{24}$N$_{10}$O$_{6}$ | 374.1033         | 374.1023       | -2.67          | 338.0816 (2f), 310.0868 | Thioli conjugation | Hydrolysis | |
The most abundant product ions are displayed in bold. The letters in parentheses indicate the proposed fragmentation. The letters a1, a2, b1, b2, c1, c2, d, and e correspond to the fragmentations indicated with these letters in Table 1. The other letters have the following proposed fragmentations: f, loss of H2O; g, loss of HPO3; h, loss of alanine; i, loss of OCNH; j, loss of CO; k, loss of HCOOH; l, loss of C3H9N; m, loss of C6H11NO2; n, loss of N-acetyl-2-iminopropanoic acid.

The similarity of the MS2 spectrum of Met15 (protonated ion at m/z 340.1418) 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).

Met21 eluted at 29.2 and 82.7 min with the 60- and 180-min HPLC methods, respectively. Because the retention times, MSn spectra, and high-resolution data of this metabolite were equal to those of the reference standard of HP1, Met21 was identified as monohydrolyzed bendamustine (HP1).

The m/z value of the protonated ion of Met22 (m/z 485) corresponded with an N-acetylcysteine (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-iminopropanoic acid) and at m/z 296.1155 (−189.045 Da, loss of N-acetyl-S-ethylcysteine).

The protonated ion of Met23, detected at m/z 356, was not included in the list of potential metabolites. The similarity of the MS5 spectrum of this metabolite with the MS2 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 37Cl isotope and by the high-resolution m/z value.

Based on comparison of the retention times, MSn fragmentation patterns, and high-resolution data with reference standards, the compounds designated Met24, Met25, and 26 were identified as γ-hydroxybendamustine (M3), N-desmethylandumestine (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 radialabeled bendamustine. Apart from the unidentified metabolite (Met1), these were the dihydrolysis products of bendamustine (Met10) and γ-hydroxybendamustine (Met6) and the cysteine conjugates of dichlorohydantoin 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

Table 3

Proposed structures of bendamustine metabolites in human urine, formed by the main metabolic conversions and arranged by structure of the benzimidazole/butyric acid moiety
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-
phate, creatinine, and uric acid conjugates; and two conjugates with unidentified moieties. Similar to mercapturic acid conjugates, thiol derivatives are common biotransformation products of cysteine conjugates (Levens et al., 2005), which can explain their presence in urine. Phosphate, creatinine, and uric acid conjugates are less commonly observed. Apart from study samples, corresponding conjugates of bendamustine were detected in control urine after incubation with bendamustine. This shows that the bendamustine-derived products containing phosphate, creatinine, and uric acid conjugates can be formed by reaction with urinary components. Although the results do not preclude the possibility that these products were formed sistematically, at least in part, they indicate that intravesical formation may be an explanation for their unexpected presence in the metabolite profile of bendamustine. Analysis of time points at which metabolites were detected in patients also indicates that their conjugation in the control urine may be primarily of bendamustine, because they were only detected in urine samples of the early time points, when bendamustine was also being excreted. Renal impairment would not affect the elimination of bendamustine because much of it is metabolized, primarily via hydrolysis, but it may decrease the intravesical formation of conjugated products and it would affect the elimination of metabolites because much of them are excreted by the kidneys.

The two metabolites with unidentified conjugations were Met12 and Met17. Met12 was a minor metabolite, only detected in the 2-h urine samples of four of the six patients and in one 6-h urine sample. The high-resolution mass of the conjugate (150.0154 Da) led to a proposed elemental composition of \( \text{C}_4\text{H}_6\text{O}_6 \) (150.0159 Da; ppm = -3.20). The product ion at \( m/z \) 408.1757 (loss of CHOOH) probably did not result from fragmentation at site e in Table 1, because the major product ion in the MS\(^3\) spectrum of \( m/z \) 408 was identified as bendamustine, split at site a1. Therefore, a carboxyl group may be 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 \( \text{C}_7\text{H}_{15}\text{NO}_3 \) (161.1046 Da; ppm = -2.48), a molecular formula corresponding with that of carnitine. This 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 \( m/z \) 424.1624, 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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