Identification and Quantitation of the N-Acetyl-L-cysteine S-Conjugates of Bendamustine and Its Sulfoxides in Human Bile after Administration of Bendamustine Hydrochloride

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ABSTRACT:

We recently reported the detection of mercapturic acid pathway metabolites of bendamustine, namely, cysteine S-conjugates in human bile, which are supposed to subsequently undergo further metabolism. In this study, we describe the identification and quantitation of consecutive bendamustine metabolites occurring in human bile using authentic reference standards and the synthesis and structural confirmation of these compounds. Mass spectrometry data along with high-performance liquid chromatography retention data (fluorescence detection) of the synthetic reference standards were consistent with those of the metabolites found in human bile after administration of bendamustine hydrochloride to cancer patients. Analysis of the purified synthetic reference compounds showed a purity of at least 95%. Structural confirmation was achieved by one- and two-dimensional proton as well as carbon-13 NMR spectroscopy and mass spectrometry. A total of 16 bendamustine-related compounds were detected in the bile of patients, 11 of them were recovered as conjugates. Eight conjugates have been structurally confirmed as novel mercapturic acids and sulfoxides. Biliary excretion of the sulfides was twice that of the mercapturate precursors. Glutathione S-conjugates of bendamustine have not been detected in bile samples, indicating rapid enzymatic cleavage in humans. Both the lack of glutathione (GSH) conjugates and occurrence of diastereomeric sulfides emphasize species-related differences in the GSH conjugation of bendamustine between humans and rats. The total amount recovered in the bile as the sum of all conjugates over the period of 24 h after dosing averaged 5.2% of the administered dose. The question of whether the novel metabolites contribute to urinary excretion should be a target of future investigations.

The anticancer agent bendamustine has been clinically used for more than 30 years in Germany as single-agent therapy or in combination with other antineoplastic drugs in a variety of dosages and regimens for the treatment of non-Hodgkin’s lymphoma, chronic lymphocytic leukemia (CLL), and multiple myeloma (Fig. 1). In March 2008, the U.S. Food and Drug Administration approved bendamustine (BM) hydrochloride for the treatment of patients with CLL. BM has a relatively mild safety profile, with myelosuppression, particularly neutropenia, being the major dose-limiting toxicity. The most common nonhematologic adverse events are vomiting, nausea, and pyrexia. In human ovarian and breast cancer cell lines, BM caused significantly more and more durable DNA double-strand breaks, resulting in lower cross-resistance compared with other alkylators such as cyclophosphamide and melphalan (Strumberg et al., 1996). In a recent study, unique mechanistic features were reported for BM compared with other alkylators that involve regulation of apoptosis pathways, inhibition of specific checkpoints during mitosis, and activation of base excision DNA repair (Gaul et al., 2008; Leoni et al., 2008). Although BM has been marketed in Germany for many years, reliable and valid information on its metabolism has only been published in recent years. The first published report of BM pharmacokinetics in humans appeared in 1985 and was based on data obtained after intravenous and oral administration of BM HCl in seven patients with tumors (Preiss et al., 1985). Because a main circulating metabolite had been detected in this study, metabolism via β-oxidation analogous to that of chlorambucil was assumed for BM. Approximately 45% of the total radioactivity administered as [14C]-labeled BM was recovered in rat bile within 24 h, indicating that biliary excretion could significantly contribute to total elimination of the drug (Bezek et al., 1991). Considerable urinary excretion of the parent compound, its hydrolysis products, and the oxidized metabolite was observed in mice (Weber et al., 1991). Several-fold higher concentrations were found in liver and kidney tissue of mice compared with those in other organs and tissues, with the highest concentrations in the gallbladder after intravenous administration of [14C]BM (Bezek et al., 1996).

ABBREVIATIONS: CLL, chronic lymphocytic leukemia; BM, bendamustine; GSH, glutathione; LC, liquid chromatography; MS, mass spectrometry; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond coherence; HSQC, heteronuclear single quantum coherence; GT, γ-glutamyl transferase; GST, glutathione S-transferase; CID, collision-induced dissociation.
Significant GSH conjugation of BM has been shown in humans as evidenced by the detection of three cysteine S-conjugates of BM in the plasma, bile, and urine of cancer patients treated with BM HCl (Teichert et al., 2005). In that study, 96 and 26% of total drug-related compounds were recovered as BM cysteine S-conjugates in the bile and urine, respectively. However, the mean percentage of the BM dose recovered in urine as the sum of parent drug, its monohydroxy and dihydroxy hydrolysis product, and five metabolites including three cysteine S-conjugates within 24 h was 8.3%, indicating that previous investigations may have overestimated renal excretion of the parent drug including both hydrolysis products. Because several peaks observed in the chromatograms remained unidentifed in that study, consecutive metabolism of the cysteine S-conjugates followed by fecal and renal excretion was expected, which warrants further examination of the metabolic pathway. More recently, considerable biliary excretion has been shown, measuring excretion of radioactive labeled BM in rats (Chovan et al., 2007). The structures of 15 novel BM metabolites have been proposed by Chovan et al. (2007) based on LC-MS fragmentation spectra of bile and urine samples from rats. Oxidative phase I metabolism was shown to be mediated by CYP1A2 as evidenced by detection of γ-hydroxy-BM and N-desmethyl-BM in human plasma, urine, and bile of cancer patients treated with BM HCl and after in vitro human liver microsomal incubations of BM HCl (Teichert et al., 2007). However, up to now the metabolic fate of BM, including the contribution of hepatobiliary and renal elimination to total elimination of BM, in humans has not been analyzed in detail. Therefore, the objective of this study was to elucidate the structure of additional metabolites formed by biotransformation of the recently described cysteine S-conjugates and to assess its contribution to total BM elimination in humans.

Materials and Methods

Materials. Reference standard 4-[5-[bis[2-chloroethyl]amino]-1-methyl-1H-benzoimidazol-2-yl]-butanoic acid hydrochloride, I (purity 99.0%), was a generous gift from Astellas Deutschland GmbH (Munich, Germany). Acetonitrile, water for HPLC, ammonium acetate, and acetic acid were purchased from J. T. Baker (Deventer, The Netherlands). N-Acetyl-L-cysteine, t-cysteine, and reduced L-glutathione were purchased from Fluka Riedel-de Haën/Sigma-Aldrich Labormedikalien GmbH (Seelze, Germany). DMSO-d6 was obtained from Chemotrade Chemiehandelsgesellschaft mbH (Leipzig, Germany). All other chemicals used in this study were purchased from Merck KgaA (Darmstadt, Germany). All reagents were of analytical grade and the solvents were of HPLC grade.

Instrumentation and Analytical Methods. Mass spectrometry. LC-MS analysis was carried out on a Finnigan SQQ-7000 single quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an electrospray ionization (ESI) interface coupled with a ConstaMetric 4100 MS Series pump, a SCM 1000 Vacuum Membrane Degasser, an autosampler AS 3000, and a model 3200 programmable wavelength detector (Thermo Fisher Scientific) coupled to a Digital Personal DEC 5000/25 workstation. ICL (version 8.3.2) and ICIS (version 8.3.0) software were used for data acquisition and data processing, respectively. HPLC separation was achieved on an Atlantis dC18 column (150 mm × 2.1 mm, 3 μm; Waters, Milford, MA) with a gradient program at a flow rate of 0.3 ml/min as follows: 0 min, 98% A (v/v); 0.2 min, 98% A; 32 min, 50% A; 34 min, 50% A; 35 min, 98% A; and 60 min, 98% A. The injection volume was 20 μl, and the mass spectrometer was operated in the ESI positive ion mode. The auxiliary and sheath gas used was Nitrogen 4.6 (Air Liquide Deutschland GmbH, Krefeld, Germany) set to 60 and 1 psi, respectively. The turbo ion spray capillary voltage was maintained at 4.5 kV and capillary temperature was set at 220°C. For each analyte, mass spectra were acquired using collision-induced dissociation (CID) with voltages of −10, −30, and −50 V. MS data were obtained by selected ion monitoring of the [M + H]+ ion of the respective BM conjugate. Data acquisition, reduction, selected ion monitoring, and peak area calculations are performed under software control by an Alpha AXP DEC 3000 Data System (Digital Equipment Corporation, Maynard, MA).

NMR spectroscropy. The 1H and 13C NMR spectra were recorded on a Mercury Plus 400 (Varian, Inc., Palo Alto, CA) and a DRX-600 (Bruker BioSpin GmbH, Rheinstetten, Germany) spectrometers at 26°C using DMSO-d6 as solvent. Residual solvent signals were used as internal chemical shift references for proton (δH(Res) = 2.49 ppm) and carbon (δC(Res) = 39.52 ppm) spectra. Signals were assigned by means of two-dimensional proton-proton (COSY) and proton-carbon (HMBC and edited HSQC) shift-correlation spectra.

Preparative HPLC. All conjugates were purified by preparative liquid chromatography using two Shimadzu LC 8 pumps (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan) equipped either with a 250 mm × 32 mm column (I) filled with HyperPrep HS C18 (12 μm, 100 Å; Thermo Fisher Scientific) or a 250 mm × 30 mm column (II) packed with Vydc 218 TPB 1520 (Grace Vydc, Hesperia, CA) at a flow rate of 32 ml/min. After dividing the reaction mixture into three aliquots, bulk separation of crude material was performed by injecting the aliquots onto column I. The mobile phase consisted of (A) 10 mM ammonium acetate solution and (B) acetonitrile-water (8:2 v/v). From 0 to 20 min, a linear gradient ran from 90% A/10% B to 20% B to remove impurities from the crude products. Main fractions were collected manually under ice cooling based on the UV signal monitored at 320 nm. Fractions from each peak, in most cases collected within 3-min periods, were concentrated under reduced pressure using a rotary vacuum evaporator. After the pH was adjusted to 3.0 by adding HCl, the concentrated fractions were applied to column II in 100- to 200-ml aliquots for isolation of impurities. The mobile phase consisted of 10 mM HCl in water (A) and acetonitrile-10 mM HCl in water (8:2 v/v) (B). The gradient was 5% to 20% B in 40 min. After manual collection, purified fractions were each rotary evaporated followed by lyophilization to dryness, yielding the respective BM conjugates as crystalline substances.

Analytical HPLC. Monitoring of products during chemical synthesis was performed on an HP 1090 gradient HPLC system with photodiode array and an HP 1046A fluorescence detector [Hewlett Packard (Palo Alto, CA), predecessor of Agilent Technologies (Santa Clara, CA)]. A column (Synergi 4 μm MAX-RP 80A, 250 × 2 mm; Phenomenex, Torrance, CA) equipped with a guard cartridge (4 × 2 mm; Phenomenex) was used for separation. The mobile phase consisted of 0.1 ml of 12 M HCl in 1 liter of water (A) and acetonitrile-water-12 M HCl (800:200:0.02 v/v/v/v) (B). The gradient was 5 to 40% B in 70 min at a flow rate of 0.3 ml/min. For analysis by photodiode array detection, UV absorption was recorded at 233 nm. Wavelengths of the fluorescence detector were set to 328 nm (excitation) and 420 nm (emission) to monitor eluted components.

Quantitation of the BM metabolites in human bile was performed with an Alliance 2695 system equipped with a fluorescence detector 2475 (Waters) coupled to a column as described above. Solvent A was 5 mM aqueous ammonium acetate (pH 3.85) adjusted with acetic acid and solvent B was acetonitrile-solvent A (80:20 v/v). The flow rate was set to 0.25 ml/min. A 10-μl injection of each sample was injected onto the column, separated, and eluted using the following gradient: 0 min, 0% B; 5 min, 6% B; 14.5 min, 8.5% B; 16 min, 14% B; 21 min, 18% B; 31 min, 30% B; 36 min, 31% B; 45 min, 40% B; 50 min, 45% B; 53 min, 2% B; and 60 min, 2% B. The column temperature and the sample temperature were maintained at 30 and 5°C, respectively. The wavelengths of the fluorescence detector were set as described above. Six-point calibration curves were fitted by weighted (1/ concentration) least-squares linear regression for each conjugate in bile using the internal standard as described previously (Teichert et al., 2005).
Synthesis and Purification of Reference Compounds. The 1-cysteine, N-acetyl-l-cysteine, and glutathione S-conjugates were synthesized using a modified procedure similar to that reported previously (Teichert et al., 2005). Accordingly, 1.3 mmol of the thiol compound was diluted in 0.4 liter of a solution containing 0.1 M sodium hydrogen carbonate and 0.2 M sodium chloride. To this solution, 0.63 mmol of the respective chloroethanol compound was added. After gentle stirring constantly at 30°C for 22 min, the mixture was cooled to 4°C in an ice bath. Alternatively, the reaction was performed at room temperature. Completion of the reaction was assessed by monitoring offline the product formation using analytical HPLC. Then, the pH was adjusted to 5.0 by adding 2 M aqueous hydrochloric acid dropwise. Product formation was monitored by analytical HPLC. Dissolved carbon dioxide gas was removed under reduced pressure before the solution was subjected to preparative HPLC as described above. For assessment of purity, the purified products were analyzed by analytical HPLC with UV detection at 233 nm after lyophilization as described under Preparative HPLC. Furthermore, each purified product was subjected to $^1$H, $^{13}$C, $^1$H-H-COSY, HMBC, and HMQC NMR as well as mass spectrometric analysis.

The following compounds were synthesized in this way: 2, 3, and 4 by reaction of 1 with glutathione, 5, 6, and 7 by reaction of 1 with l-cysteine, 7 by further reaction of 16 with l-cysteine, 8 by reaction of 9 with l-cysteine, 9, 10, and 11 by reaction of 1 with N-acetyl-l-cysteine, 11 by further reaction of 16 with N-acetyl-l-cysteine, and 14 by reaction of 12 with l-cysteine. Compounds 12 and 13 were synthesized by oxidation of 9 and 11, respectively, as follows: 250 ml of 10 mM ammonium acetate solution containing a 0.5 mM fraction containing the oxidized product was added to a solution of the respective compound was adjusted to pH 4.8 by addition of 2 M HCl. To this solution, 5 ml of 30% hydrogen peroxide solution were added while stirring. The mixture was kept under stirring at room temperature and the oxidation progress was monitored by analytical HPLC. At a maximal product yield, the reaction was stopped and the mixture was subjected to preparative HPLC and worked up in the usual way.

Compound 15 was obtained from 5 by a two-step synthesis. The first step of the synthesis was the reaction of 5 with hydrogen peroxide as described for compounds 12 and 13. After purification by preparative HPLC, the collected fraction containing the oxidized product was added to a solution of N-acetyl-cysteine in 0.1 M sodium hydrogen carbonate and 0.2 M sodium chloride as detailed above for compounds 2 through 14 and 17. The final product was obtained as a crystalline solid after preparative HPLC and lyophilization as described above.

Compound 16 was obtained by hydrolysis of 1 as follows: 0.5 mol of 1 hydrochloride diluted in 2 ml of dimethyl sulfoxide were added to a 120 mM aqueous solution of sodium hydrogen carbonate and the mixture was kept under stirring for a further 45 min at 30°C. The reaction was stopped by cooling on ice and adding 2 M HCl. After the pH was adjusted to 3.0, the diluted carbon dioxide was removed under reduced pressure. A subsequent workup procedure of the reaction mixture was performed as described for the other compounds. To prove identity of the products, minor amounts of the compounds 5, 9, and 12 were converted into the compounds 7, 11, and 13, respectively, using the method described for synthesis of 16.

Characterization of Reference Compounds. Purity of reference compounds was determined by HPLC, and major MS fragment ions are reported with the most abundant fragment ion produced at a CID voltage of 50 V presented in bold type.

<table>
<thead>
<tr>
<th>Reference compound</th>
<th>Retention time (min)</th>
<th>M/z [M + H]$^+$</th>
<th>M/z [M + Cl]$^+$</th>
<th>M/z [M + CHO]$^+$</th>
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<td>2</td>
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<td>501.01</td>
<td>474.99</td>
<td>[M + H]$^+$</td>
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<td>528.09</td>
<td>501.01</td>
<td>474.99</td>
<td>[M + H]$^+$</td>
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<tr>
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<td>4.5</td>
<td>528.09</td>
<td>501.01</td>
<td>474.99</td>
<td>[M + H]$^+$</td>
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<tr>
<td>5</td>
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<td>501.01</td>
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<td>[M + H]$^+$</td>
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<tr>
<td>6</td>
<td>4.5</td>
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<td>501.01</td>
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<td>[M + H]$^+$</td>
</tr>
<tr>
<td>7</td>
<td>4.5</td>
<td>528.09</td>
<td>501.01</td>
<td>474.99</td>
<td>[M + H]$^+$</td>
</tr>
</tbody>
</table>

Glutathione conjugates 2, 3, and 4. The typical fragmentation processes well known for glutathione conjugates were observed for the compounds 2 through 4. Cleavage of the C-S bond was observed at either side of the sulfur atom. If the bond was cleaved within the cysteine moiety, the charge resided on the tertiary nitrogen for each of the metabolites photographed concomitant with the loss of the sulfur of cysteine occurred concomitant with the loss of the carbon dioxide moiety.
Occurrence of the fragment ion \( m/z \) 234 in the spectrum obtained for metabolite 6 indicates that cysteine S-conjugation appears to facilitate cleavage of the C–N bond. The molecular ions were the most intensive peaks for CID voltages up to 30 V. No parent molecular ion was observed for the cysteine S-conjugates increasing the CID offset to 50 V, and heterolytic fragmentation of the S-CH₂ bond between the cysteine moiety and the diethylamino group of BM with charge retained on the sulfur generating a sulfinium (\( R - S^+ \)) ion was apparent as evidenced by the most abundant fragment ion at \( m/z \) 120. The abundance of the cysteine fragment ion (\( m/z \) 120) in compounds 5 through 7 was much higher than that of any of the other conjugates.

Mercapturic acid conjugates 8, 9, 10, and 11. The mercapturic acid conjugates undergo the fragmentations discussed for the cysteine conjugates. Moreover, the occurrence of the N-acetylcysteine moiety was confirmed by cleavage of the S-CH₂ within the cysteine moiety leading to a neutral loss of 129 Da, corresponding to acetylamino propionic acid that was found abundantly in the 50 V CID spectra of all mercapturic acid conjugates. The corresponding fragment ion was observed at \( m/z \) 130. In addition, the mercapturic acids yielded the abundant fragment ion at \( m/z \) 162 assumed to be N-acetylcysteine-sulfenium ion. The structure of the N-acetylcysteine S-conjugate 8 showed a fragmentation pattern different from those of the monocysteine conjugates of BM, yielding an abundant fragment ion at \( m/z \) 423 formed by loss of the cysteine-S-ethyl group.

Mercapturic acid sulfoxides 12, 13, 14, and 15. The fragmentation results from MS analysis were consistent with the structures assumed for the mercapturic acids oxidized at the sulfur atom of either the cysteine or the N-acetylcysteine moiety. As with all cysteine conjugates, the sulfoxides exhibited fragment ions by cleavage at either side of the sulfur. If two sulfur atoms are present in the molecule, more abundant fragment ions were observed by cleavage at the sulfide sides. In particular, for compound 15, parallel losses of 2-amino-propionic acid (alanine) and the N-acetylcysteine-S-ethyl moiety resulted in the most intensive fragment ion at \( m/z \) 310 corresponding to BM ethylsulfenic acid followed by a loss of methanesulfenic acid similar to the fragmentation known for the monooxidized thioether bond in the cysteinyl side chain of a peptide under low-energy MS conditions. For fission of the opposite carbon–sulfoxide bond as observed by neutral losses of cysteine sulfenic acid and the N-acetylcysteine-S-ethyl moiety, the resulting ethylamino radical of BM can be stabilized by aziridine formation, yielding a fragment ion at \( m/z \) 260. The fragmentation pattern included further fragment ions at \( m/z \) 130 as well as at 162 as already described for the mercapturic acids. On the one hand, general lability of the sulfoxides was observed in low-voltage CID mass spectra as indicated by significantly less abundant molecular ions compared with the spectra of the other conjugates. On the other hand, mass spectral data revealed large differences in the intensities of the molecular ions between the mono- and dicysteine sulfoxides. The least abundant molecular ions were observed for the monacysteine sulfoxides 12 and 13. In Fig. 2, the mass spectra of the structurally isomeric sulfoxides 14 and 15 are presented.

Some BM S-conjugates were complex and partially overlapping. However, the spectra obtained from the reference compounds by two-dimensional NMR analyses do fit the anticipated structures. The NMR data for compounds 5 through 7 were detailed in our preceding article (Teichert et al., 2005). The chemical shifts for the other reference standards observed in the proton and carbon NMR are closely related to those for the cysteine S-conjugates 6, 8, and 7 and are summarized in Tables 1, 2, and 3. Moreover, \(^{1}H\) and \(^{13}C\) NMR spectra of the oxidized cysteine conjugates of BM, yielding an abundant fragment ion at \( m/z \) 423 formed by loss of the cysteine-S-ethyl group obtained by synthesis was not sufficient to perform NMR experiments. There was a significant difference of 4.4 min in the HPLC retention times between \( \gamma \)-hydroxy-BM mercapturic acid and the structural isomeric conjugate 12, both with molecular ions at \( m/z \) 501. Moreover, a split peak was observed in the chromatogram of \( \gamma \)-hydroxy-BM mercapturic acid, giving the strong implication that the reaction product was a mixture of a pair of diastereomers. The second chiral center in the molecule is generated by \( \gamma \)-hydroxylation of the butyric acid side chain. To facilitate characterization of the metabolite structures, the chlorine isotope peaks at \( m/z \) 501.2 and \( m/z \) 503.2 containing the chlorine isotopes \(^{35}\)Cl and \(^{37}\)Cl, respectively, were mass-analyzed by LC-tandem mass spectroscopy in 0.1-atomic mass unit increments from 10 atomic mass units through the mass of the precursor ion. The characteristic fragment ions at \( m/z \) 162 and 130, which have also been observed after cleavage of the C–S binding at either side of the sulfur in the N-acetylcysteine conjugates, clearly indicate that there was no sulfoxide present in the reaction product of N-acetyl-L-cysteine with \( \gamma \)-hydroxy-BM (19). This characteristic fragmentation pattern did not occur in the constitutionally isomeric sulfoxides as demonstrated by fragmentation of metabolite 12.

Preparation of Stock Solutions and Calibration Samples. Individual stock solutions of the reference standards were prepared by dissolving adequate amounts of the conjugates in acetonitrile after structural confirmation to obtain final concentrations of approximately 1.0 mg/ml and were stored at \(-20^\circ\)C. Samples for calibration curves were prepared by adding standard solutions obtained by appropriate dilution of the respective stock solution with 0.01 M hydrochloric acid to 1 ml of blank bile. This yields calibration standard concentrations in the range of approximately 0.1 to 100 \( \mu \)mol/ml.

Sample Preparation for Quantitative Analysis. Aliquots of 0.05 ml of the working solution of the internal standard were added to bile samples from subjects (0.05 ml each) and diluted with 0.9 ml of 0.01 M aqueous hydrochloric acid, centrifuged at 15,000 \( \times \) g for 5 min. The solution was then filtered through a 0.2-\( \mu \)m Rotilabo nylon supported membrane filter from Carl Roth GmbH (Karlsruhe, Germany). A 10-\( \mu \)l aliquot of the filtrate was injected into the HPLC system.

Subjects and Sampling Procedure. Samples were obtained from five subjects with cholangiocarcinoma after intravenous infusion of BM hydrochloride solution. The intravenous dose received by each patient on day 1 of the first of four cycles overall was 140 mg/m². Two temporary external nasobiliary drainage tubes (7-French, 290 cm, 8 holes; Endo-Flex, Voerde, Germany) were placed into the left and right hepatic ducts, each with endoscopic retrograde cholangiography and left in place for the entire collection period. Bile was collected quantitatively before dosing and during 16 intervals up to 24 h after the 30-min infusion was started. Ten-milliliter aliquots of the bile samples were stored in polypropylene tubes at \(-70^\circ\)C until measurement. The nasobiliary drainage tubes were removed, and permanent endoscopic stenting was performed after sample collection. The Ethics Committee of the Faculty of Medicine of the University of Leipzig issued approval for this clinical trial.

Results

Characterization of BM Metabolites Observed in Human Bile. The metabolites detected in the bile are summarized in Fig. 3. The conjugates detected in the bile were designated according to their chronological order of formation followed by the hydrolysis products and phase I metabolites. A total of 16 substances were identified in human bile, 5 of which were assigned as previously identified parent drug (1) and two hydrolysis products thereof, namely, monohydroxy as well as dihydroxy-BM (16 and 17) and two oxidative metabolites (18 and 19). The 11 remaining metabolites were identified as cysteine S-conjugates (5–7) and their N-acetylated (mercapturic acid) as well as N-acetylated and oxidized (mercapturic acid sulfoxide) forms (8–15). Identification of the metabolites was confirmed by comparison of their retention times and molecular as well as fragment masses on HPLC with fluorescence detection and mass spectra, respectively, with those of the synthesized reference substances. In addition, metabolite 12 was isolated from the bile by preparative HPLC and subsequently subjected to LC-MS analysis. The chromatogram and MS fragmentation pattern at low CID voltage of metabolite 12 isolated from human bile confirmed the results obtained by
HPLC as well as LC-MS analysis of the respective synthetic reference standard, indicating that this metabolite was BM mercapturic acid sulfoxide. The presence of two diastereomers for each of the sulfoxides 13 and 14 was confirmed in the bile samples by HPLC analysis showing two distinctly separated peaks. Their retention characteristics were identical to those for the peaks detected after chromatographic separation of the reference standards 13 and 14, obtained by chemical synthesis. Sufficient chromatographic separation achieved for the pairs of highly polar diastereomers such as 13 and 14 diminished with increasing retention time, leading to asymmetrical peak shape for the pairs of nonpolar diastereomeric sulfoxides. However, the chromatographic conditions had been established to guarantee sufficient separation and quantitation of 16 drug-related compounds from the biliary matrix.

Metabolic Profile of BM in Human Bile and Recovery of the Administered Dose. The BM metabolites 5 through 15 recovered in human bile were determined quantitatively using HPLC with fluorescence detection. Our recently reported results concerning the cysteine S-conjugates have been confirmed by measuring the highest individual maximal concentrations for the conjugates 5 and 7, accounting for 0.35 and 0.32 mM, respectively. Maximal concentrations measured for the conjugates 8, 11, 12, and 13 were 5- to 10-fold lower. Representative chromatograms of bile samples collected from subjects after infusion of 140 mg/m² BM hydrochloride are presented in Fig. 4. Individual concentrations of BM conjugates in the bile reached a peak level approximately 60 to 90 min after drug administration and declined over a period of 5 to 6 h with the exception of the sulfoxides, which showed prolonged...
The bile acids and sulfoxides. The time course for biliary excretion of the BM metabolites monitored over 24 h is shown in Fig. 5. Table 4 shows the mean amounts of BM-related compounds excreted into the bile of cancer patients over the 24-h collection period after infusion of BM HCl solution. An average of 5.2% of the dose given to the subjects was cumulatively excreted into bile as conjugates over a period of 24 h. Unchanged parent drug excreted through bile accounted for 0.03% of the administered dose in the same period. No glutathione S-conjugates of BM were detected in bile specimens collected from patients (Fig. 6).

### Table 1

Proton NMR data for the reference compounds 2 through 4, 8, 14, and 15

<table>
<thead>
<tr>
<th>Proton</th>
<th>2</th>
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<th>8</th>
<th>14</th>
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<td>8.51 (1H, t)</td>
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<td>8.35 (1H, d)</td>
<td>8.38 (1H, d)</td>
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<tr>
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<td>3.49 (3H, s)</td>
<td>3.50 (3H, s)</td>
<td>3.56 (3H, s)</td>
<td>3.87 (3H, s)</td>
<td>3.82 (3H, s)</td>
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</table>

| NH₄Cl    | 3.78 (2H, t) | 3.73 (4H, b) | 3.73 (2H, b) | 3.78 (2H, b) | 3.74 (2H, b) | 3.74 (2H, b) |
| CH₃Cl    | 3.74 (2H, t) | 3.74 (2H, t) | 3.74 (2H, t) | 3.74 (2H, t) | 3.74 (2H, t) | 3.74 (2H, t) |

Cys, cysteine; GS, glutathione-S; Het, heterocyclic; br, broad; splitting patterns denoted as s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet.

### Table 2

Proton NMR data for the reference compounds 9 through 13

<table>
<thead>
<tr>
<th>Proton</th>
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<th>11</th>
<th>12</th>
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<td>H-4</td>
<td>7.68 (1H, d)</td>
<td>7.67 (1H, d)</td>
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<tr>
<td>H-3</td>
<td>7.06 (1H, d)</td>
<td>7.05 (1H, d)</td>
<td>7.04 (1H, d)</td>
<td>7.37 (1H, d)</td>
<td>7.37 (1H, d)</td>
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<tr>
<td>H-1</td>
<td>6.84 (1H, d)</td>
<td>6.81 (1H, d)</td>
<td>6.83 (1H, d)</td>
<td>6.96 (1H, d)</td>
<td>6.94 (1H, d)</td>
</tr>
<tr>
<td>CH₂NH</td>
<td>4.39 (1H, m)</td>
<td>4.39 (1H, m)</td>
<td>4.43 (1H, m)</td>
<td>4.64 (1H, m)</td>
<td>4.64 (1H, m)</td>
</tr>
<tr>
<td>NCH₂</td>
<td>3.88 (3H, s)</td>
<td>3.87 (3H, s)</td>
<td>3.87 (3H, s)</td>
<td>3.89 (3H, s)</td>
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<tr>
<td>SOCH₂CH₂</td>
<td>3.76 (2H, s)</td>
<td>3.76 (2H, s)</td>
<td>3.77 (2H, s)</td>
<td>3.77 (2H, s)</td>
<td>3.77 (2H, s)</td>
</tr>
<tr>
<td>CH₃Cl</td>
<td>3.76 (2H, s)</td>
<td>3.76 (2H, s)</td>
<td>3.77 (2H, s)</td>
<td>3.77 (2H, s)</td>
<td>3.77 (2H, s)</td>
</tr>
<tr>
<td>N-Acetyl-Cys SCH₂CH₂</td>
<td>3.76 (2H, s)</td>
<td>3.76 (2H, s)</td>
<td>3.77 (2H, s)</td>
<td>3.77 (2H, s)</td>
<td>3.77 (2H, s)</td>
</tr>
</tbody>
</table>

Cys, cysteine; Het, heterocyclic; br, broad; splitting patterns denoted as s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet.

a Not assigned either due to broadening or signal overlapping.
Moreover, we have elucidated the structure of the novel mercapturic acids and confirmed their structures as BM mercapturic acid oligates in the human bile of the patients treated with BM HCl as well. We have detected three of these metabolites in the bile of patients treated with BM HCl, which have been proposed on the basis of their mass spectral fragmentation. In a recent study, the structures of 15 novel metabolites of BM in rats have been proposed on the basis of their mass spectral fragmentation and NMR analyses. GSH conjugation of various alkylating agents has been shown by in vitro experiments but has not been shown as yet by in vivo identification in humans after alkylator exposure. The results of the present study are accord with the findings of early as well as recent animal experiments in rats using radioactive labeled BM, which have shown significant biliary excretion as indicated by recoveries of 45 and 50% of the administered radioactivity in the bile and feces, respectively (Bezek et al., 1991; Chovan et al., 2007).

In a recent study, the structures of 15 novel metabolites of BM in rats have been proposed on the basis of their mass spectral fragmentation (Chovan et al., 2007). We have detected three of these metabolites in the human bile of the patients treated with BM HCl as well and confirmed their structures as BM mercapturic acids. Moreover, we have elucidated the structure of the novel mercapturic acid and four sulfoxides, occurring in human bile, indicating cytochrome P450-mediated sulfoxidation of the mercapturic acids and.

Syntheses of the novel sulfoxides by nonspecific sulfur oxidation with hydrogen peroxide yielded enantiomerically pure diastereomers attributable to the chiral sulfoxide derived from the enantiomerically pure (R)-sulfoxide and the enantiomerically pure (R)-sulfides and, respectively. Occurrence of pairs of diastereomers of the conjugates and in human bile was demonstrated by comparing the chromatograms of the bile samples with those of mixtures of enantiomerically pure diastereomeric sulfoxides obtained by synthesis. Chromatographic conditions used in this study were not capable of separating the diastereomers of compound and, therefore, no conclusion can be drawn with respect to its formation in vivo.

Furthermore, we obtained a mixture of diastereomeric y-hydroxy-BM mercapturic acid by reaction of y-hydroxy-BM (19) with N-acetyl-l-cysteine. The retention time difference between the peaks of y-hydroxy-BM mercapturic acid and the sulfoxide was approximately the same as reported for the peaks at m/z 501 in rat urine and, therefore, no conclusion can be drawn with respect to its formation in vivo.

Results obtained in this study provide evidence, on the one hand,
that there are no fundamental species differences in metabolism of BM as shown by structural elucidation of several metabolites that are identical or similar to those recently proposed for rats after administration of BM HCl. Chovan et al. (2007) stated that the mercapturic acid 10 was the major biliary metabolite in rats, accounting for approximately 60% of total peak area. We found only minor amounts of that metabolite in human bile, whereas the cysteine S-conjugates, mercapturates and its sulfoxides, occurred as major components. Furthermore, in apparent contradiction to the results in rats, the glutathione S-conjugates, 2, 3, and 4 were not detectable in human bile. All together, 11 BM-derived conjugates have been quantitatively determined in the human bile in this study, indicating the relative importance of the GSH conjugation pathway in the metabolism of BM in humans. Four of the conjugates are novel sulfoxides. On the other hand, this result confirms the species difference between rats and humans concerning γ-GT-dependent biliary-hepatic recycling. In the rat and mouse, which have relatively low levels of hepatic γ-GT, cysteine S-conjugates are preferentially formed in the intestine and kidney bearing high concentrations of γ-GT (Hinchman et al., 1991). Guinea pigs and humans can readily cleave glutathione S-conjugates because of relatively high concentrations of γ-GT in the biliary tree.

BM did not exhibit complete cross-resistance with other alkylators, for example, chlorambucil and melphalan. In human ovarian and breast carcinoma cell lines, the degree of resistance to BM was lower compared with that of cyclophosphamide, melphalan, and N,N’-bis(2-chloroethyl)-N-nitrosourea. Moreover, BM induced more extensive and long-lasting DNA double-strand breaks than other alkylators (Strumbo et al., 1996). Growth inhibitory activities of melphalan,
chlorambucil, and the phosphoramide mustard of cyclophosphamide strongly correlated with each other in a broad variety of human tumor cell lines using the National Cancer Institute in vitro antitumor screen, but no significant correlation was obtained between these anticancer agents and BM (Leoni et al., 2008). Remission rates of more than 55% in phase I/II studies with rather small sample sizes of 16 and 15 demonstrated good response to multicycle treatment with BM in patients with relapsed or refractory B-cell lymphocytic leukemia (CLL), who had been heavily pretreated with chlorambucil (Bergmann et al., 2005; Lissitchkov et al., 2006). Encouraging remission rates ranging from 73 to 83% were reported for BM after multicycle therapy in more than 100 patients with alkylator (chlorambucil, melphalan, and cyclophosphamide)-refractory low-grade non-Hodgkin’s lymphomas (Heider and Niederle, 2001; Bremer, 2002). An overall response of 77% was obtained in 76 patients with rituximab-refractory predominantly stage III/IV indolent B-cell non-Hodgkin’s lymphoma. Moreover, lymphomas in 30% of these patients had been refractory to a recent alkylator-based regimen (Friedberg et al., 2008).

The reason for these encouraging results with BM achieved in patients with alkylator-refractory lymphoid malignancies has not yet been fully explained. Besides the alkylating activity of the nitrogen mustard, possible antimitabolite activity has been discussed because of the benzimidazole heterocycle of BM (Konstantinov et al., 2002; Schwanen et al., 2002; Leoni et al., 2008). However, this pharmacodynamic profile should be verified and further substantiated. Differences in cellular GSH-mediated detoxifying mechanism may also confer differences in therapeutic response to BM compared with other alkylators, mainly chlorambucil and melphalan. Results from our own unpublished studies with recombinant human GSTs showed that formation of monoglutathione S-BM is mediated by GSTA 1-1 rather than by GSTM 1-1 but not by GSTP 1-1. In agreement with these results, GSH conjugation of chlorambucil is substantially mediated by GSTP 1-1 (Pandya et al., 2000; Zhang and Hangan, 2003; Parker et al., 2008), which is the main GST isoenzyme in lymphoid tissues (Bennaceur-Griscelli et al., 2004). Therapeutic response and resistance to therapy of BM and chlorambucil in non-Hodgkin’s lymphoproliferative malignancies are currently under investigation by our group.

In conclusion, eight novel metabolites of the GSH conjugation pathway of BM, which were structurally confirmed as mercapturates and the corresponding sulfoxides of BM are described herein. The proposed metabolic pathway of BM includes a total of 18 metabolites, including the previously characterized hydrolysis products, phase I metabolites, and cysteine S-conjugates. The latter represent the major biliary metabolites of BM. Further studies should focus on renal excretion of the conjugates described herein to fully clarify their contribution to total clearance of BM in humans.
TABLE 4

Total amounts of the compounds excreted into bile during 24 h after i.v. administration of 140 mg/m² BM hydrochloride in five patients with cancer

<table>
<thead>
<tr>
<th>Amount</th>
<th>1</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.D.</td>
<td>0.06</td>
<td>4.93</td>
<td>1.00</td>
<td>6.32</td>
<td>0.39</td>
<td>0.41</td>
<td>0.15</td>
<td>1.64</td>
<td>1.25</td>
<td>3.47</td>
<td>0.57</td>
<td>0.11</td>
<td>0.11</td>
<td>0.25</td>
<td>0.14</td>
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</table>

**References**


