Isolation and Structure Elucidation of Urinary Metabolites of Mitoxantrone

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ABSTRACT

Three 13C-labeled 1,4-dihydroxy-5,8-bis[2-(2-hydroxyethyl)amino]ethy]amino-9,10-anthracenedione dihydrochloride (mitoxantrone) isotypomers were synthesized to prove the proposed chemical structures of human urinary metabolites by means of nuclear magnetic resonance spectroscopy. After application of labeled mitoxantrone to an anesthetized pig, urine was collected by way of a vesicourethral catheter. The urinary metabolites were isolated by liquid chromatography using a new procedure developed for extraction of mitoxantrone metabolites. Structural elucidation by nuclear magnetic resonance spectroscopy and tandem mass spectrometry confirmed the proposed mono- and dicarboxylic acid structures of the metabolites. High-performance liquid chromatography analyses showed that the new urinary metabolite represented the main biotransformation pathway of mitoxantrone in pigs, indicating significant interspecies variation in mitoxantrone biotransformation. Expressed in equivalents of mitoxantrone, the new metabolite amounts to 25% and 31%, respectively, of urinary excreted drug-related material. Extraction of patient urine using the same procedure led to the isolation of pure metabolite B. Tandem mass spectrometric data delivered definitive evidence for the structure of metabolite B as monocarboxylic acid of mitoxantrone.

INTRODUCTION

Mitoxantrone (Novantrone, NSC 301739; American Cyanamid Company), (Fig. 1, mitoxantrone M) is a representative of synthetic anticancer agents developed to diminish cardiotoxicity associated with the clinical application of anthracyclines (1-3). Although the drug is widely used in treatment of breast cancer, acute leukemia, and non-Hodgkin’s lymphoma, little is known about its metabolism in humans and animals.

During the investigation of the pharmacokinetics of the drug in patients, only 2 metabolites of mitoxantrone, 1,4-dihydroxy-5,8-bis[2-(1-carboxymethyl)amino]ethyl]amino]-9,10-anthracenedione (Fig. 1, metabolite A) and 1,4-dihydroxy-5,8-bis[2-(1-carboxymethyl)amino]ethyl]amino]-8-[2-(2-hydroxyethyl)amino]ethyl]amino]-1,10-anthracenedione (Fig. 1, metabolite B) have been detected in plasma and urine (4-14). They were identified as drug-related material by typical light absorption of the anthracenedione chromophor or by radioactivity after application of 13C-labeled drug. Incubation of human urine with β-glucuronidase or sulfatase did not alter the retention times of these metabolites in high performance liquid chromatography, suggesting that they are neither glucuronide nor sulfate conjugates of mitoxantrone (14).

In rabbits (15) and rats (16, 17), however, additional metabolites were observed, and in vitro experiments with rat liver microsomes and hepatocytes indicated that mitoxantrone might conjugate with glucuronic acid and glutathione (9, 18, 19). The structures of the mitoxantrone conjugates found in animals are still unknown, and the structures of the human metabolites require further investigation because the published spectrometric data are not sufficient for final structural proof. The main reason for the poor knowledge of the metabolites’ structures is related to the very low occurrence of urinary metabolites, the lack of suitable methods for preparative separation, and problems in mass spectrometric detection of the parent compound and the metabolites in urinary extracts (20).

The chemical structures of the 2 human urinary metabolites have been proposed in this journal in 1986 by coworkers of the American Cyanamid Company (6) (Fig. 1, A and B). Both metabolites have also been detected in rabbits (15) and pigs (20). Structure elucidation was achieved by mass spectrometry using negative ions. However, chemical ionization using methane as the reagent gas led to very low intensities of negatively charged molecular ions, especially in the case of the metabolite regarded as monocarboxylic acid derivative (metabolite B). Thus, the proposed oxidation of the terminal hydroxymethylene groups of the side chains remains to be proved using methods more suitable for the identification of carboxylic acids.

This report describes a new analytical technique for the extraction of mitoxantrone metabolites from urine. The method has been developed to protect metabolites from chemical denaturation during extraction and separation. Mass spectrometric analyses of the highly purified metabolites have been carried out using soft ionization methods such as electrospray and fast atom bombardment ionization. Both methods produce high intensities of molecular ions of the metabolites. Structure elucidation has been carried out by application of tandem mass spectrometry and led to the unequivocal identification of 2 urinary metabolites.

To obtain further evidence for the oxidation of the side chains, 13C-labeled mitoxantrones were synthesized (Fig. 2). After application to pigs, metabolites could be isolated from urine. The structural changes in the side chain(s) could be confirmed by 13C-NMR spectroscopy.

MATERIALS AND METHODS

Drugs and Reagents

Reagents. Sodium [13C]cyanide with 91% 13C enrichment was purchased from Amersham-Buchler (Braunschweig, Federal Republic of Germany). [1-13C]Glycine with 99% 13C-enrichment and β-glucuronidase (EC 3.4.1.31) was obtained from Sigma (Deisenhofen, Federal Republic of Germany). Synthetic reference substances of metabolites A and B were kindly supplied by Lederle Laboratories (Pearl River, NY). Sodium 1-pentanesulfonate (98%) and dimethylformamide (HPLC grade) were purchased from Aldrich (Steinheim, Federal Republic of Germany).
In Vivo Experiments

In Vivo Experiments

German, acetonitrile (HPLC grade) from Merck (Darmstadt, Federal Republic of Germany) and Baker (Gross-Gerau, Federal Republic of Germany), 1-pentanesulfonic acid (Pic B5) from Waters Millipore (Eschborn, Federal Republic of Germany). Water was deionized and filtered with a Milli-Q water purification system (Milford, MA). PRP-1 resin (11-20 pm) was purchased from Hamilton (Darmstadt, Federal Republic of Germany), and LiChrosorb RP-select B (10 pm) was from Merck. Other chemicals and reagents were obtained from regular commercial sources.

Metabolite Isolation

Urine was centrifuged in fractions of 800 ml at 3000 rpm for 20 min and then filtered through a hollow fiber dialyzer to remove solid impurities and high molecular weight substances (especially peptides) to avoid damage to the chromatographic columns (dialysis was only necessary when human urine was processed). After dialysis, solid sodium 1-pentanesulfonate was added to achieve a 5 mM urinary concentration. The addition was necessary to avoid partial loss of the metabolites due to desorption by urinary components during the solid phase extraction with PRP-1 resin. After rinsing the column with 50 ml of a 1 mM aqueous solution of sodium 1-pentanesulfonate, the metabolites were separated using a step gradient from 5 mM aqueous solution of sodium 1-pentanesulfonate and acetonitrile (10:90, v/v). All fractions were lyophilized immediately and stored at room temperature until further purification. The fraction containing metabolite C was resolved in water and chromatographed on LiChrosorb RP-select B with a linear gradient from 0 to 20% of solvent B with 5 mM aqueous solution of sodium 1-pentanesulfonate and acetonitrile (20:80, v/v) as solvent B. The fractions containing mitoxantrone in high purity were lyophilized, resolved in 2 ml of water, and adsorbed on a disposable filtration column (Baker) filled with 1 ml PRP-1 to remove pentanesulfonic acid. After the column was washed with 4 ml water, mitoxantrone was desorbed with 0.1 M methanolic hydrochloric acid. Purity of the product was 99.9% (determined by HPLC). The product was lyophilized immediately and stored at 4°C.

In Vivo Experiments

Experiments were performed in male Göttinger minipigs weighing 25 kg. Atropine (0.5 mg), azapenone (100 mg), ketamine hydrochloride (29 mg), and flunitrazepame (2 mg) were used as sedatives before anesthesia with isoflurane/oxygen. A laparotomy was performed and the bile duct was cannulated. Urine was collected by way of a vesicourethral catheter. For venous access, the vena cava was catheterized. A few minutes before application, mitoxantrone was dissolved in 20 ml 0.3% sodium chloride solution. The drug was injected as a bolus via the vena cava catheter. Urine and bile were collected up to 8 h after application of the drug. During the experiment, 3 liters of electrolyte solution and 10 mg furosemide (30 min after application of the drug) were applied by continuous infusion via the catheter.

High-Performance Liquid Chromatography

The chromatographic system for preparative isolation of metabolites from urine consisted of a HPLC pump equipped with a preparative pumphead (model 2200; Bischoff, Leonberg, Federal Republic of Germany). For urine extraction, glassy columns (Superperformance system, 50x16 mm; Merck) were used. The columns were filled in our laboratory with 4 g PRP-1 resin. Depending on the quality of the urine, up to 2 liters of urine could be extracted with one column. The urine was pumped on the column with a flow rate of 10-20 ml/min. Due to the intense blue color of mitoxantrone and its metabolites, the different fractions could easily be detected visually.

Further purification of the metabolites was achieved with a gradient HPLC pump (model 2249) and an integrator (model 2221; Pharmacia-LKB, Freiburg, Federal Republic of Germany). Light absorption was measured with a Spectra 200 UV-visible detector (Spectra-Physics, Darmstadt, Federal Republic of Germany). Injection was carried out by hand using Hamilton syringes and a Rheodyne 7125 injection valve on an ECB/Switching valve (VICI; Schenkon, Switzerland). Glassy columns (150x10 mm) filled with LiChrosorb RP-select B were used at flow rates of 1-2 ml/min. Analytical separations were obtained using C18+Bondapak columns (300 x 3.9 mm; 10 pm; Waters Millipore, Eschborn, Federal Republic of Germany).

Quantitation of mitoxantrone and metabolites in native urine and bile was performed by means of a HPLC apparatus equipped with a Waters pump 510, a Waters 712 sample processor (WISP), and a Waters Lambda-Max model 481 LC spectrophotometer. The Maxima software from Waters was used for data evaluation.

Metabolite Isolation

Urine was centrifuged in fractions of 800 ml at 3000 rpm for 20 min and then filtered through a hollow fiber dialyzer to remove solid impurities and high molecular weight substances (especially peptides) to avoid damage to the chromatographic columns (dialysis was only necessary when human urine was processed). After dialysis, solid sodium 1-pentanesulfonate was added to achieve a 5 mM urinary concentration. The addition was necessary to avoid partial loss of the metabolites due to desorption by urinary components during the solid phase extraction with PRP-1 resin. After rinsing the column with 50 ml of a 1 mM aqueous solution of sodium 1-pentanesulfonate, the metabolites were separated using a step gradient from 5 mM aqueous solution of sodium 1-pentanesulfonate and acetonitrile (10:90, v/v). All fractions were lyophilized immediately and stored at room temperature until further purification. The fraction containing metabolite C was resolved in water and chromatographed on LiChrosorb RP-select B with a linear gradient from 0 to 20% of solvent B within 20 min (solvent A was a mixture of 5 mM aqueous solution of sodium 1-pentanesulfonate, solvent B was a mixture of 5 mM aqueous solution of sodium 1-pentanesulfonate and acetonitrile (20:80, v/v)). The metabolite fraction was lyophilized, resolved in 2 ml water, and adsorbed on a disposable filtration column filled with 300 μl PRP-1 resin. To remove sodium 1-pentanesulfonate, the column was washed with 10 ml water and the metabolite was desorbed with methanol (90:10, v/v).

Most fractions were sufficiently pure, and only removal of excess sodium 1-pentanesulfonate was necessary. In the case of metabolite A, several irregularities were observed. Even with more than 40% methanol, only partial desorption could be achieved. A purple part remained. Furthermore, a mixture of methanol and 0.1 M hydrochloric acid (1:1, v/v) failed to elute the bulk of adsorbed metabolite. Elution could be effected by treatment with 2 M NaOH, and the color turned back to blue using basic conditions. Few fractions had to be purified using a newly developed HPLC separation because interfering urinary components could not be eliminated using the HPLC conditions described above. Separation was effected using a PRP-1 filled glassy column and a basic buffer (0.1 M sodium carbonate, pH 9.1) containing
Nuclear Magnetic Resonance Spectroscopy

NMR data were obtained on a W/M 400 and AM 600 NMR spectrometer from Bruker (Rheinstetten, Federal Republic of Germany). 

\(^{13}\)C-NMR spectra of 3'-\(^{13}\)C- and 4'-\(^{13}\)C-labeled metabolites were acquired using the \(^1\)H-broadband decoupling technique. Two-dimensional NMR spectra of 1'-\(^{13}\)C-labeled metabolites were measured using a pulse sequence allowing extremely high sensitivity for \(^{13}\)C-labeled positions by making use of polarization transfer (22). The data acquisition occurred in the region of protons. Splitting of the resonance peaks into doublets with a coupling constant of \(J_{CH}\) is the consequence. The 2-dimensional heteronuclear shift correlation NMR experiment allows the correlation of carbon atoms bearing a proton. Carbons without an adjacent proton were not detected and had to be measured using normal \(^1\)H-broadband decoupling.

Mass Spectrometry

For acquisition of fast atom bombardment and chemical ionization mass spectra, a triple stage quadrupole mass spectrometer TSQ 70 from Finnigan-MAT (Bremen, Federal Republic of Germany) was used. IonSpray mass spectra were obtained with an API III mass spectrometer of Sciex (Thornhill, Ontario, Canada). For details of the ionization techniques, see the literature (23, 24).

Enzymatic Degradation

Metabolite C was incubated with \(\beta\)-glucuronidase in an ammonium acetate buffer that was adjusted to pH 5 with acetic acid. After 40 min at 37°C, HPLC analyses revealed quantitative liberation of mitoxantrone.

RESULTS AND DISCUSSION

Figs. 3 and 4 illustrate the HPLC chromatogram of native urine and native bile, respectively, from pigs treated with mitoxantrone. The urinary excretion of mitoxantrone and its metabolites was studied in 4 pigs. Quantitation results are summarized in Tables 1 and 2. Metabolite C, up to now not observed in human urine, was the main excretory pathway within the observation period of 5 h. Metabolites A and B showed the same retention times as the known human urinary metabolites and were produced in the same range as found in patients. Therefore, minipigs are more suitable for investigation of mitoxantrone metabolism than rats that do not produce the 2 urinary metabolites\(^4\) observed in urine of humans. As previously found in humans, mitoxantrone and metabolites with unknown structures are mainly excreted via bile (25). Biliary excretion of pigs contains mainly mitoxantrone and metabolite B and only very small amounts of the other metabolites found in the urine. The similar behavior of metabolite B compared with mitoxantrone is consistent with the presence of one unchanged side chain in the biotransformation product B. The isoelectric point of mitoxantrone was determined by isoelectric focusing to 9.15.\(^4\) Thus, the basic amino group of the side chain remains protonated even in bile (pH 8.2). As discussed later, metabolite C represents a glucuronic acid derivative of mitoxantrone, whereas metabolite A has the structure of a dicarboxylic acid. Therefore, both should form anions at the pH of bile. A strong relation between ion charge and pathway of excretion can be observed.

Identification of urinary components as drug-related material

URINARY METABOLITES OF MITOXANTRONE

Fig. 4. HPLC chromatogram (detected at 658 nm) of 10 μl pig bile (dilution 1:10) after i.v. injection of 6.5 mg [3-'C]mitoxantrone/kg. Separation was achieved on a C18-Bondapak column using isocratic conditions and 5 mM pentanesulfonic acid/acetonitrile (76:24, v/v) as solvent system.

Table 1 Urinary excretion of mitoxantrone and metabolites after i.v. injection of different [13C]mitoxantrone isotopomers

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Urine (ml)</th>
<th>Mitoxantrone</th>
<th>Metabolite C*</th>
<th>Metabolite A*</th>
<th>Metabolite B*</th>
<th>Time* (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1-'C</td>
<td>4</td>
<td>540 6.9</td>
<td>0.17</td>
<td>0.08</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2 1-'C</td>
<td>935 1.1</td>
<td>0.14</td>
<td>0.4</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 3-13C</td>
<td>6.5 1800</td>
<td>0.89</td>
<td>0.33</td>
<td>0.26</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>4 4-'C</td>
<td>52 610</td>
<td>1.33</td>
<td>0.30</td>
<td>0.43</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

* Quantitation was performed by injection of 1 ml native urine.

Table 2 Biliary excretion of mitoxantrone and metabolites after i.v. injection of [3-13C]mitoxantrone

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Bile (ml)</th>
<th>Mitoxantrone</th>
<th>Metabolite C*</th>
<th>Metabolite A*</th>
<th>Metabolite B*</th>
<th>Time* (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>44.2</td>
<td>8.3</td>
<td>0.15</td>
<td>1.2</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

* Quantitation was performed by injection of 10 μl 1:10 diluted bile.

B correspond well with data obtained from the reference substances in our laboratory. Structure elucidation of metabolite C was also effected by NMR spectroscopy. Fig. 6 shows the 2-dimensional heteronuclear shift correlation NMR spectra of [1-'13C]mitoxantrone demonstrating the high sensitivity for ['3C labeling. NMR-data of [4-'13C]mitoxantrone and metabolite C are given in Table 4. The structure of metabolite C is illustrated in Fig. 7.

From the data presented in Table 3, it follows that in metabolites B and C, the 2 formerly identical side chains have become different. The small differences in the chemical shifts of the 2 terminal carbon atoms (C-4', C-4''') in metabolite C (Table 4) indicate 2 slightly different chemical environments. Furthermore, the 2 chemical shifts are very close to the value found for C-4' in mitoxantrone (Table 3).

All 13C-signals of the unlabeled positions are due to the natural 13C-abundances (1.1%) and therefore, compared with the 2 labeled positions, are relatively weak. In particular, the chemical shift assignments of quartenary carbon atoms may be less exact. From the enzymatic degradation experiment and from mass spectrometric studies, it follows that metabolite C is a glucuronic acid conjugate of the parent mitoxantrone. Either glucuronidation at a phenolic hydroxyl or at an alcoholic hydroxyl could produce molecular asymmetry responsible for the splitting of the 13C-labeled terminal carbon atoms. From the 13C-NMR data, no decision between these 2 possibilities can be made. However, in the 1H-NMR spectrum, the 4 aromatic protons at C-2/3 and C-6/7 are split into 4 doublets. The observed splitting pattern suggests glucuronidation at a phenolic rather than an alcoholic hydroxyl. Details of the 1H-NMR spectrum will be given in a forthcoming publication.

The large downfield shift of the resonance of the terminal carbon atom in one side chain of metabolite B indicates the conversion of a hydroxymethylene group into a carboxylic group.

Efforts to isolate metabolites from urine were hindered by the absence of procedures allowing preparative separation. Thus, the development of a new extraction and separation method was necessary to allow the extraction of the metabolites of mitoxantrone from urine in a very efficient way. Extraction by ion pairing with sodium 1-pentanesulfonate was found to give very good recovery, but retention of the metabolites was found to be pH dependent and substantial losses of metabolite A have been noticed at pH >6. This behavior suggests that basicity of the side chain amino groups is reduced after biotransformation because retention of the metabolites during extraction depends on the interaction of protonated substrate and the ion-pairing reagent used. The oxidation of C-1 of the 2-aminoethanol moiety yields an N-alkylated amino acid with strongly decreased basicity of the amino group. The equilibrium between the different forms in which amino acids could exist is shifted at higher pH to neutral or negatively charged forms lacking pairing possibilities with the ion-pairing reagent used. As urinary pH was 5–6, buffering was not necessary to obtain good recoveries of oxidized metabolites. The rather unusual behavior of metabolite A to precipitate from eluate and to be extremely poorly soluble in most solvents and neutral aqueous solutions made it difficult to obtain MS and NMR spectra. Low solubility and changes of color during separation are probably a result of the zwitterionic structure of the side chain after biotransformation. The purple color observed during separation could be explained as the result of protonation of the

Table 3 13C-NMR chemical shifts of the various 13C-labeled carbons in the side chains of mitoxantrone and from metabolites isolated from pig urine

<table>
<thead>
<tr>
<th>Chemical shift δ [ppm]</th>
<th>[1-'13C]</th>
<th>[3-'13C]</th>
<th>[4-'13C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sidechain</td>
<td>1 + 2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>41.3°</td>
<td>51.8°</td>
<td>51.8°</td>
</tr>
<tr>
<td>Metabolite C</td>
<td>40.5°</td>
<td>52.2°</td>
<td>52.2°</td>
</tr>
<tr>
<td>Metabolite A</td>
<td>41.5°</td>
<td>50.5°</td>
<td>50.5°</td>
</tr>
<tr>
<td>Metabolite B</td>
<td>39.4°</td>
<td>51.8°</td>
<td>51.8°</td>
</tr>
</tbody>
</table>

Solvents: * deuterium oxide; ° deuterium oxide/deuterohydrochloric acid; ° dimethyl sulfoxide-de°, ° dimethyl sulfoxide-de°/deuterohydrochloric acid.
URINARY METABOLITES OF MITOXANTRONE

Fig. 5. 13C-NMR spectra of mitoxantrone and metabolite A isolated from pig urine after application of [4'-13C]mitoxantrone. M. 13C-NMR spectrum of synthesized [4'-13C]mitoxantrone. A. 13C-NMR spectrum of 4'-13C-labeled metabolite A.

Fig. 6. Two-dimensional heteronuclear shift correlation NMR spectra of 1'-13C-labeled metabolite C.

phenylenediamine moiety of the chromophor causing a hypochromic shift of light absorption. In the zwitterionic form, these amino groups are more basic than those of the amino acid moiety of the side chain.

In our view, the mass spectrometric data obtained by Chiccarelli et al. (6) cannot be seen as rigorous structural proof for metabolite B. The chemical ionization technique used in their study is known to produce adduct ions (26, 27). With methane as the reagent gas, the negative molecular ions produced are often accompanied by a series of ions of the composition [M + (CH3)n]. In fact, control measurements with mitoxantrone showed the occurrence of negative ions having higher masses than the molecular ion at m/z 444. Accidentally, these ions at m/z 458 and 472 (n = 1, n = 2) interfere with the molecular masses of metabolites A and B.

The daughter ion spectra of negatively charged 5,8-diamino-1,4-dihydroxy-9,10-anthracenediones obtained by application of the mass spectrometry/mass spectrometry daughter scan mode are dominated by an intense fragment ion at m/z 268. Using the parent scan mode of our triple stage quadrupole mass spectrometer, it is possible to demonstrate the occurrence of adduct ions of mitoxantrone by detection of parent ions of m/z 268 (Fig. 8). The presence of the ion m/z 444, which is not a fragment ion of pure metabolite B, indicates the presence of mitoxantrone in the sample measured by Chiccarelli et al. (6).

Thus, the ion at m/z 458 regarded as molecular ion of metabolite B could also represent one of the adduct ions of mitoxantrone described above.

Fig. 9 illustrates the daughter ion spectra of 13C-labeled metabolite A and B isolated from pig urine and the corresponding daughter ion spectra of the unlabeled reference substances. The collision-activated dissociation mass spectra of metabolite B isolated from patient urine shows the identity of metabolite B in humans and pig (Fig. 10). The ions of m/z 459 and 473 produce the same characteristic fragments after collisional activation as the corresponding reference substances. This proves that these ions in fact represent the positively charged molecular

<table>
<thead>
<tr>
<th>Table 4</th>
<th>13C-NMR data of the glucuronic acid conjugate of mitoxantrone (solvent, D2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resonance</td>
<td>ppm</td>
</tr>
<tr>
<td>Carbon: 1a</td>
<td>1.42, 2.3, 5.8, 6.7</td>
</tr>
<tr>
<td>δ (ppm): 152.5, 128.3, 148.2, 127.4</td>
<td>—, —</td>
</tr>
<tr>
<td>Carbon: 1′′′′, 2′′′′, 3′′′′, 4′′′′</td>
<td>1′, 2′′, 3′′, 4′′, 5′′, 6′′</td>
</tr>
<tr>
<td>δ (ppm): 41.3, 49.2, 52.2, 59.77, 177.9, 75.5, 72.2, 77.9, 74.5, 105.1</td>
<td></td>
</tr>
</tbody>
</table>
*Resonances are not detectable.

Fig. 7. Structure of metabolite C (molecular mass, 620 dalton).

Fig. 8. Negative ion collision-activated dissociation mass spectra of mitoxantrone after detection of parents of the 5,8-diamino-1,4-dihydroxy-9,10-anthracenedione typical fragment ion 268. Adduct ions with methylene radicals (458 and 472) were observed above the 444 molecular mass of mitoxantrone (ionization: desorption chemical ionization with methane as reagent gas; source temperature, 150°C; manifold temperature, 70°C; source pressure, 0.5 Torr; collision gas, argon; collision gas pressure, 2.5 m Torr; collision energy, 50 eV).
Fig. 10. Collision-activated dissociation mass spectra of metabolite B (m/z 459) isolated from patient urine showing identity of metabolite B in humans and pig (ionization, IonSpray; collision gas, argon; collision gas pressure, $3.6 \times 10^{-4}$ atm/cm²; orifice potential, 80 V; collision chamber potential, −50 V).

Fig. 11. Proposed collision-activated dissociation fragmentation pathway of positive ions of 3'-¹³C-labeled mitoxantrone and 3'-¹³C-labeled metabolites.

Fig. 12. Collision-activated dissociation mass spectra of [3'-¹³C]mitoxantrone (M, [M+H]^+ m/z 447) and the [3'-¹³C]glucuronic acid conjugate of mitoxantrone (C, [M+H]^+, m/z 623) obtained with IonSpray ionization at atmospheric pressure (collision gas, argon; collision gas pressure, $3.7 \times 10^{-4}$ atm/cm²; orifice potential, 100 V; collision chamber potential, −50 V).

ions of the mono- and dicarboxylic acid of mitoxantrone as suggested by Chiccarelli et al. (6). Fragmentation patterns of the molecular masses are explained in Fig. 11.

Mass spectra of metabolite C have been acquired using
IonSpray ionization. Structure elucidation of metabolite C is supported by the daughter ion spectra of the $^{13}$C-labeled metabolite, which is shown in Fig. 12. The loss of neutral dehydro-glucuronic acid from the protonated molecular cation [MH-176]$^+$ has been reported to be a favored fragmentation pathway of ether and phenol-linked glucuronides (28) and is also observed in the daughter ion spectra of metabolite C. Further fragmentation is typical for the mitoxantrone moiety. HPLC analyses after incubation of metabolite C with β-glucuronidase prove that mitoxantrone is liberated from the metabolite.

Human biliary excretion of mitoxantrone metabolites is well established (25), but the structures of biliary metabolites are still unknown. Although molecular mass threshold values for biliary excretion are assigned to be about $470 \pm 50$ g/mol in humans and $400 \pm 50$ in guinea pigs (29), the glucuronic acid conjugate with a molecular mass of 620 is excited predominantly via urine. This pathway of biotransformation may represent a new detoxification pathway for mitoxantrone in humans if human liver shows different behavior in excretion selectivity.

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