Identification of Human Urinary Mitoxantrone Metabolites


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ABSTRACT

Two polar metabolites of mitoxantrone, a clinically active antitumor agent, have been isolated and purified from the urine of patients by sequential absorption on glass wool and C18-Sep-Pak cartridges followed by preparative high-performance liquid chromatography. Negative ion chemical ionization mass spectrometry indicated that the two metabolites are the di- and mono-carboxylic acids resulting from oxidation of the terminal hydroxyl groups of the side chain(s). Mass spectral comparison of the urinary metabolites with synthetic compounds confirmed the identification.

INTRODUCTION

Mitoxantrone (Novantrone; American Cyanamid Company), 1,4-dihydroxy-5,8-bis-[[2-[(2-hydroxyethyl)amino]-ethyl]amino]-9,10-anthracenedione dihydrochloride, NSC 301739, is an anticancer drug which has shown significant activity during Phase II and III clinical trials (2-4). Mitoxantrone was among a large series of compounds synthesized in the American Cyanamid laboratories by Murdock et al. (5). Zee-Cheng and Cheng (6) reported the activity of some bis-substituted aminoalkylaminoanthraquinones including the subject compound, confirming observations of the efficacy of mitoxantrone against transplanted murine tumors (7). Pharmacokinetic studies in humans (8-13) and animals (14-16) together with the analytical methodology (17-21) developed during these evaluations have been reported. The characteristics of drug-related material in patient urine (1, 8, 9) and animal bile and urine (22-25) have been described.

Following i.v. administration of [14C]mitoxantrone to human patients, in one study (8) 6.5% of the drug was found in the urine after 5 days at a total urinary radioactivity of 10.1%. In a second study 7.3% of the drug was excreted in the urine after 3 days; the total radioactivity was 11.3% (13). That the urine was a minor excretory pathway for parent drug was also confirmed in our laboratory from our assay results from the urine of pediatric patients given a single dose of unlabeled mitoxantrone (26). However, direct HPLC injection of the urine samples established the presence of two polar metabolites in addition to mitoxantrone. Here we report the isolation, purification, identification, and synthetic techniques used to characterize the two human urinary metabolites.

MATERIALS AND METHODS

Reagents and Material

Formic acid and ammonium formate were purchased from Fisher Scientific Co. (Fairlawn, NJ). C18-Sep-Pak cartridges were obtained from Waters Associates (Milford, MA). Glass wool was obtained from American Scientific Products (Edison, NJ). Partisil PXS CCS/C4 reversed phase analytical and preparative columns and the Spheri RP-GU guard columns were obtained from Whatman, Inc. (Clifton, NJ) and Brownlee Labs, Inc. (Santa Clara, CA), respectively. All chemicals were of reagent grade; solvents were spectral grade. Administered mitoxantrone was better than 97% on an anhydrous basis (HPLC). [14C]Mitoxantrone, labeled in the carbon atoms α to the arylamino groups, was 94.9% radiochemically pure (HPLC). Urine samples had been collected in ice-chilled plastic containers and either frozen (8, 26) or refrigerated until extracted and analyzed.

High-Performance Liquid Chromatography

A Partisil PXS CCS/C4 analytical (4.6-mm x 25-cm) or preparative (9.4-mm x 50-cm) column fitted with a Spheri RP-GU guard column was used. The mobile phase, 1.0 M ammonium formate (pH 4.3):acetonitrile (3:1), was filtered (0.45 μm) and degassed before use. A Waters Associates M-45 solvent delivery system and 441 absorbance detector operated at 658 nm with manual injection was used.

Metabolite Isolation

Urine was diluted with 1 volume of water and passed dropwise through a loosely packed glass wool column (0.5 x 15 inches) which was then washed with water. Drug components were eluted as a blue band with 0.1 M methanolic HCl. After accumulation of several runs, the combined eluates were diluted with 1 volume of water and concentrated by passing through C18-Sep-Pak cartridges (conditioned with methanol and water) until saturated. After a water wash the drug material was eluted with 1-3 ml of 0.001 M methanolic HCl.

Another procedure consisted of passing mitoxantrone patient urine through C18-Sep-Pak cartridges (prewashed with water) which were linked serially in pairs. After saturation, the cartridges were washed with water. Drug components were then eluted with methanol-water, starting with a mixture containing 40% methanol and followed by mixtures having 5% methanol increments. Portions of each methanol eluate were analyzed by HPLC. Since mitoxantrone and the two polar metabolites are blue, the HPLC elution profiles and colors were compared in order to select the methanol eluate with the greatest metabolite recovery potential.

After extraction from urine by either of the above procedures, preparative HPLC was performed and the metabolites were separated by fractional collections. Each was removed from the mobile phase by passage through prewashed C18-Sep-Pak cartridges. Formate was removed by a water wash and the metabolite was eluted from the cartridge with 1-3 ml 0.001 M methanolic HCl. The separated metabolite fractions were concentrated to 0.5 ml under a stream of nitrogen and cooled to 5°C. Metabolite A gave a precipitate which was examined by MS; metabolite B remained in solution and the solution was used for the MS analysis.

Radiochemical Techniques

Radioactivity was determined with a Beckman LS-9000 liquid scintillation spectrometer (Beckman Instrument Co., Fullerton, CA) and the absolute radioactivity was computed by a sample channels ratio calibration procedure. HPLC eluates were counted directly in Prewblend 3a70B scintillation cocktail (Research Products International Corp., Elk Grove Village, IL).

Spectrophotometry

A purified concentrate of the urinary drug-related material was separated by the HPLC procedure with the eluate monitored by on-line...
Synthesis of Metabolites

Metabolites A and B were synthesized by a method similar to that reported by Murdock et al. (5). Thus, reaction of 2,3-dihydro-1,4,5,8-tetrahydroxy-9,10-anthracenedione with tert-butyl 2-aminoethanol formate, followed by oxidation with chloranil and acid hydrolysis, gave the dicarboxylic metabolite B. Similarly, the monocarboxylic metabolite A was prepared by sequential treatment of 2,3-dihydro-1,4,5,8-tetrahydroxy-9,10-anthracenedione with 2-(2-aminoethoxy)ethanol and tert-butyl 2-aminoethanol formate, followed by oxidation and hydrolysis. The structures of the synthetic compounds A and B were established both by the method of synthesis and from spectroscopic and analytical data.

Synthetic Metabolite A (Glycine, N,N'-[(9,10-Dihydro-5,8-dihydroxy-9,10-dioxo-1,4-anthracenediyI)bis(imino-2,1-ct hancdiy 1 )|his-, Dihydrochloride]. IR (KBr): 2940 (NH2 stretch), 2800-2400 (NH, OH, NH2+), 1740 (C=O of COOH), 1605 and 1670 (C=O of quinone), 1560 (aromatic ring stretch), 1458 (CH2 deformation), 1200 (aryl O and CNC vibration), 810 (aromatic H out-of-plane bending) cm⁻¹. H-NMR: 1.16 (s, 6H, D6), 3.69 (m, 2H, OCH2), 3.20 (m, 4H, NCH2s), 3.05 (m, 2H, CH2OH), 2.85 (t, 2H, NH2+), 7.70 (d, J = 9 Hz, 1 H, H3), 7.69 (d, J = 9 Hz, 1 H, H2), 7.22 (s, 2H, H6, H7), 3.94 (m, 6H, aryl NCH2x2 and NCH2COO), 3.69 (m, 2H, OCH2), 3.20 (m, 4H, NCH2x2), 3.05 (m, 2H, NCH2), ppm. C-NMR (DMSO-d6): 183.8 (2, C9, C10), 167.8 (2, C=O of COOH), 154.6 (2, C5, C8), 145.9 (2, Cl, C4), 124.7 (2, C6, C7), 124.5 (2, C2, C3), 114.2 (2, C11, C12), 108.1 (2, C13, C14), 46.9 (2, NCH2COOx2), 45.6 (2, NCH3x2), 38.3 (2, aryl NCH3x2) ppm.

C22H24N4O7-2HCl.H2O
Calculated: C 46.90, H 5.01, N 9.94
Found: C 46.95, H 5.23, N 9.70

Synthetic Metabolite B (Glycine, N-[2-[(9,10-Dihydro-5,8-dihydroxy-9,10-dioxo-1,4-anthracenediyI)bis(imino-2,1-ethanediyI)]bis-, Dihydrochloride]. IR (KBr): 2960 (MI. stretch), 2800-2400 (NH, OH, NH2+), 1740 (C=O of COOH), 1610 and 1670 (C=O of quinone), 1565 (aromatic ring stretch), 1460 (CH2 deformation), 1200 (aryl O and CNC vibration), 810 (aromatic H out-of-plane bending) cm⁻¹. H-NMR: 9.83 (l, CH2N), 47.0 (l, NCHjCOO), 45.8 (2, NCH2x2), 38.4 (2, aryl NCH3x2) ppm.

C22H26N4O7-2HCl.H2O
Calculated: C 46.90, H 5.01, N 9.94
Found: C 46.95, H 5.23, N 9.70

RESULTS AND DISCUSSION

Fig. 1 illustrates a HPLC chromatogram obtained by direct injection of a patient's urine containing a high proportion of the more polar metabolites (A and B). The patient received an i.v. dose of 12 mg mitoxantrone/m². The urine from pediatric patients (26) when subjected to the same HPLC treatment in our laboratory exhibits the same qualitative elution profile.

Urine collected from patients dosed with [14C]mitoxantrone was obtained and was found to contain the same two polar metabolites. Fig. 2 shows the HPLC 658 nm chromatogram and radioactivity elution profile of a urine sample. Comparison of the two chromatograms indicates that the radioactivity resides in the two polar metabolites as well as in mitoxantrone.

Fig. 1. HPLC chromatogram (detection at 658 nm) of 0-24 h patient urine after an i.v. dose of mitoxantrone at 12 mg/m². Urine, 200 μl, was injected directly into a Partisil PXS CCS/C4 column at an elution rate of 1.3 ml/min at a normal pressure of 1200 psi. A and B, metabolites A and B (see Fig. 4).

Fig. 2. HPLC chromatogram at 658 nm (A) and radioactivity elution profile (B) of 0-24 h mitoxantrone-treated patient urine. The patient received a single i.v. dose of [14C]mitoxantrone (4.83 μCi/mg), 12 mg/m², with the total dose equal to 22 mg mitoxantrone (8). A 200-μl aliquot of the urine was injected into the column at an elution rate of 1.3 ml/min at a normal pressure of 1200 psi.
Samples of the metabolites (A and B) from each of two patients were purified and isolated for MS. Comparisons of the spectra of each metabolite with those of the synthetic acids (Fig. 3, C and D) show that metabolites A and B are the diamino-carboxylic acids resulting from oxidation of the terminal hydroxy groups of the side chain(s) of mitoxantrone (Fig. 4). The major urinary metabolite (A) has a MS fragmentation pattern indicative of the dicarboxylic acid, M, 472, with a M⁻ 472⁻, a base peak at 428⁻ (loss of CO₂), and small peaks at 414⁻, 384⁻, 371⁻, 356⁻, and 327⁻ which are consistent with fragments which can be expected from the dicarboxylic acid structure. The minor urinary metabolite (B) has a MS fragmentation pattern indicative of the monocarboxylic acid, M, 458, with M⁻ 458⁻, a base peak at 414⁻ (loss of CO₂), and several peaks which are consistent with fragments which can be expected from this structure. Differences in certain of the ion intensity ratios between the synthetic and metabolic preparations (Fig. 3) are attributable to the matrix effects observed in chemical ionization spectra (28). These effects are due to differences in the matrix of endogenous components, such as moisture and urinary and chemical impurities. Certain ions in the metabolites, not seen in the synthetic preparations, were shown to be unrelated to the metabolites by examination of ion chromatograms which showed that the extraneous ions were not coelutes of metabolite ions (i.e., the m/z 468⁻ in Fig. 3B).

The spectral characteristics of the two urinary metabolites determined by on-line diode array spectrophotometry are in agreement with the above structures. A purified concentrate of the urinary drug-related material was separated by the HPLC procedure with eluate channeling into the sample cell of the diode array spectrophotometer. The superimposed spectra of the separated drug-related components shown in Fig. 5 are similar and did not show a shift in absorption maxima. This indicates that the two metabolites have the same 1,4-diamino-anthrquinione chromophore present in mitoxantrone. Macpherson et al. (29) and Smyth et al. (30) have also reported the presence of this chromophore in two human urinary metabolites of mitoxantrone, although the complete structures were not established.

Synthetic samples of metabolites A and B were both inactive when tested against P388 leukemia in mice, a test procedure in which mitoxantrone exhibits high activity (7). Therefore, metabolites A and B are regarded as metabolic detoxification products.

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REFERENCES

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