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Note

Determination of metronidazole and misonidazole and their metabolites in plasma and urine by high-performance liquid chromatography

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The nitroimidazole derivatives, metronidazole and misonidazole are currently being investigated for their ability to sensitize hypoxic cells to radiation damage as well as for their selective toxicity against hypoxic cells [1—3].

Quantitative correlations between nitroimidazole cell concentrations and radiation enhancement have been demonstrated [4]. This finding, along with the known central nervous system toxicity [5] and potential carcinogenicity [6] make it imperative to monitor drug levels in plasma and urine.

Analytical methods currently available utilizing UV spectrophotometry [7] and polarography [8] do not differentiate between these drugs and their potentially active nitroimidazole metabolites. A previously reported gas—liquid chromatography—electron capture assay [9] is sensitive and specific for the parent drug but too lengthy for routine use. This report presents a reversed-phase high-performance liquid chromatography (HPLC) method with direct analysis of deproteinized serum and urine and UV detection at 324 nm. This procedure is specific for metronidazole or misonidazole and their metabolites with the unchanged nitroimidazole moiety.

MATERIALS AND METHODS

Apparatus and reagents

A Waters Assoc. (Milford, Mass., U.S.A.) OT757 high-performance liquid chromatograph was used, equipped with a Model 6000A pump, UK6 injector and reversed-phase μBondapak C_{18} column (30 cm × 4 mm I.D., average par-

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ticle size 10μm). Eluents were monitored by UV absorbance at 324 nm utilizing a Spectroflow monitor model SF770 from Schoeffel Instrument Co. (Westwood, N.J., U.S.A.). The HPLC eluent was 8% acetonitrile in 10^{-3} *M* phosphate buffer, pH 4.0. The flow-rate was 2.0 ml/min.

Mass spectra were obtained on an AEI-901 chemical ionization (isobutane) mass spectrometer (Kratos Ltd., AEI, Manchester, Great Britain) using direct insertion.

Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole], misonidazole [1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol] and desmethylmisonidazole [1-(2-nitro-1-imidazolyl0-2,3-propanediol] were obtained from the National Cancer Institute (Bethesda, Md., U.S.A.). All chemicals were of analytical reagent grade quality.

PROCEDURE

Serum and diluted (1:2 in water) urine samples were mixed with equal volumes of ethanol, vigorously shaken for 10 sec and proteins allowed to precipitate for 15 min at room temperature. The mixture was then centrifuged at 1700 *g* for 10 min and 20 μl of the supernatant were injected onto the HPLC column. Peaks eluting from the column were quantitated using peak heights and compared with an external standard curve at three different drug concentrations.

RESULTS AND DISCUSSION

Metronidazole and misonidazole were not separable on the C_{18} reversed-phase column under the experimental conditions. As little as 5 ng of these drugs were detectable when using UV absorption at 324 nm. The direct serum and urine assay after deproteinization without further purification steps was sufficiently sensitive for pharmacological studies. The sensitivity of the assay is about 0.5 μg/ml for both drugs when 10 μl of serum or urine are analyzed. The sensitivity limit can be further lowered by injecting larger volumes since biological background is negligible. None of the control or patient samples showed any interfering peaks.

Typical HPLC records obtained from human urine and serum samples are shown in Fig. 1 which includes analysis of a blank serum and a serum sample to which misonidazole and its major metabolite desmethylmisonidazole (10 μg/ml each) have been added. Standard curves of misonidazole, desmethylmisonidazole and metronidazole are linear from 0.5 μg/ml to at least 100 μg/ml serum. Precision was determined for misonidazole and desmethylmisonidazole at 10 μg/ml and 20 μg/ml respectively and gave $\bar{X}$ = 9.94 μg/ml (S.D. 0.15; C.V. 1.5%; n = 5) for misonidazole and $\bar{X}$ = 20.19 μg/ml (S.D. 0.45; C.V. 4.5%; n = 5) for desmethylmisonidazole.

Chromatograms obtained from serum and urine samples of patients receiving oral doses of 1 g/m^2 misonidazole and 6 g/m^2 metronidazole are also included with Fig. 1. It can be noted that misonidazole is more concentrated than its metabolite desmethylmisonidazole in serum (Fig. 1C) of which the opposite is true in urine (Fig. 1D). This indicates a higher renal clearance for the more po-

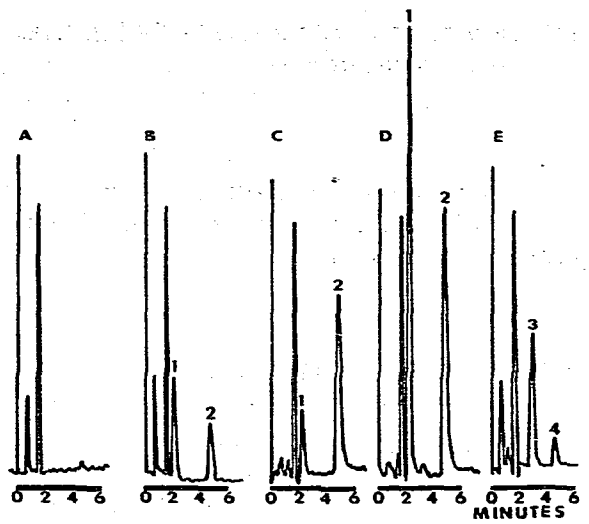

Fig. 1. Misonidazole and metronidazole HPLC records. (A) Drug-free patient serum; (B) drug-free serum spiked with 10 μg/ml of misonidazole and desmethylmisonidazole; (C) serum sample collected 12 h after a dose of 1.0 g/m² misonidazole (misonidazole 28 μg/ml; desmethylmisonidazole 6 μg/ml); (D) diluted (1:2) urine sample collected 15 h after a dose of 1.0 g/m² misonidazole (misonidazole 68 μg/ml; desmethylmisonidazole 60 μg/ml; (E) serum sample collected 27 h after a dose of 6 g/m² metronidazole (metronidazole 2.8 μg/ml and approximately 10 μg/ml of metronidazole metabolite. Peaks: 1=desmethylmisonidazole; 2=misonidazole; 3=metronidazole; 4=metronidazole metabolite.

lar desmethyl metabolite. The urine sample was rechromatographed by HPLC on the same column using the aqueous eluent buffer without acetonitrile in order to separate desmethylmisonidazole from potentially present glucuronide metabolites. Only a single peak was observed with a retention time identical to that of authentic desmethylmisonidazole (7.2 min) indicating absence of further metabolites.

A patient serum sample taken 27 h following a metronidazole dose of 6 g/m² (Fig. 1E) gave a metronidazole concentration of 14.0 μg/ml using the previously described UV assay [7] and 2.8 μg/ml by HPLC. The discrepancy between these assays can be accounted for by the level of a major metabolite (Fig. 1E), which was analyzed by mass spectrometry. The collected HPLC fraction corresponding to this metabolite of metronidazole was purified by silica gel column chromatography (eluent chloroform—methanol, 10:1) and analyzed by chemical ionization mass spectrometry. A quasi-molecular ion, *m/e* 188, indicated the insertion of one oxygen atom into metronidazole. A major hydroxylated metabolite of metronidazole (M.W. 187) has been previously isolated from human urine as the only metabolic species corresponding to the addition of one oxygen atom [10]. We therefore conclude the identity of the material isolated by HPLC to be 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole.

No standard curve was constructed for the quantitation of this metabolite due to the lack of an appropriate reference compound. The ratio between metronidazole and its metabolite varied considerably between patients.

The presented HPLC method will be utilized to determine the pharmacoki-

netics of misonidazole and metronidazole in patients undergoing radiation therapy in conjunction with these nitroimidazole radiosensitizers.

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