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Note

Sample preparation and a liquid chromatographic assay for misonidazole and desmethylmisonidazole*

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The nitroimidazoles, which include misonidazole (Fig. 1), are potent hypoxic cell radiosensitizing compounds with good correlation between the nitroimidazole serum concentrations and radiation enhancement [1]. The toxicity of these compounds for the central nervous system [2] coupled with their possible carcinogenicity [3] complicates their use. Measurement of serum levels of the compounds to assure adequacy for the desired radiosensitization while minimizing neuropathogenic levels can improve health-care safety standards for the radiation enhancement procedure.

A review of the literature for methods to measure the specific nitroimidazoles indicated that liquid chromatographic methods would be the optimal approach. The high-performance liquid chromatographic (HPLC) method of Marques et al. [4] gave us poor resolution after the ethanol serum sample clean-up procedure. In addition the method did not have an internal standard. The HPLC methods of Workman et al. [5] and Meering and Maes [6] which measure misonidazole and its demethylated metabolite utilize a large volume of methanol per volume of serum for the sample clean-up, which reduces the minimum detectable limits. Neither of the methods [5, 6] could separate misonidazole from metronidazole, and we wanted to use the latter compound as the internal standard. Meering and Maes [6] double the analytical run time to get their internal standard in the assay. None of these authors [4—6] give any summarized patient serum levels of misonidazole or desmethylmisonidazole based on dosage per body surface area.

We describe a study of the effectiveness and volumes of solvent required of

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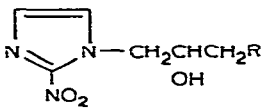
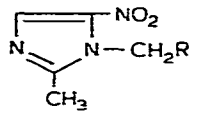


MISONIDAZOLE		R = OCH_3
DESMETHYLMISONIDAZOLE		R = OH
Ro 07-0741	(IS)	R = F
Ro 07-0913		R = OCH_2CH_3
Ro 07-0269		R = Cl



METRONIDAZOLE	R = CH_2OH
Ro 11-6924	R = COOH
Ro 07-0207	R = $CH(OH)CH_2Cl$

Fig. 1. Structural formulae of misonidazole and metronidazole and their related nitroimidazole derivatives.

methanol, ethanol, acetonitrile and methanol—acetonitrile (3:1) for the amount of protein removed from serum samples. The effects of these solvents are also described with regard to sample solvent and volume that is acceptable for column injection on reversed-phase columns. We have been able to reduce the volume of serum required by 80% for the measurement of misonidazole and desmethylmisonidazole in comparison to other authors [5, 6], and to remove 99.6% of the serum proteins prior to the analytical step. We have a four-fold increase in sensitivity with this assay as compared to the method of Meering and Maes [6]. We summarize patient serum levels in relation to the drug dose given based on body surface area.

EXPERIMENTAL

Reagents

We obtain acetonitrile and methanol, both HPLC grade, and potassium phosphate monobasic from J.T. Baker, Phillipsburg, NJ, U.S.A. Absolute ethanol (200 proof) is obtained from U.S. Industrial Chemicals, New York, NY, U.S.A. Water is deionized by repeated passage through a four-cartridge (organic removal, two mixed-bed ion-exchangers, and a 200 μm pore exclusion) filter system (Barnstead Nanopure; Barnstead/Sybron, Boston, MA, U.S.A.). The water supplied to this unit is from a commercial deionizer.

Desmethylmisonidazole, misonidazole and metronidazole are obtained from the National Cancer Institute (Bethesda, MD, U.S.A.) and used as received. The following nitroimidazoles are supplied by Hoffmann LaRoche, Nutley, NJ, U.S.A.: 1-(2-nitroimidazol-1-yl)-3-fluoropropan-2-ol (Ro 07-0741), 1-(2-nitroimidazol-1-yl)-3-ethoxypropan-2-ol (Ro 07-0913), 1-(2-nitroimidazol-1-yl)-3-chloropropan-2-ol (Ro 07-0269), 1-(2-methyl-5-nitroimidazol-1-yl)-3-chloropropan-2-ol (Ro 07-0207, and 1-(2-methyl-5-nitroimidazol-1-yl)-acetic acid (Ro 11-6924).

Chromatography

The ambient temperature chromatography is performed with a Model 6000A

solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model 7120 injection valve and a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.). The Ultrasphere ODS (octadecylsilane, 5 μm) (150 × 4.6 mm) packed column is obtained from Altex Scientific, Berkeley, CA, U.S.A. The Varian MCH-10 10-μm (300 × 4.0 mm) (MCH is a monomeric octadecylsilane packing), MCH-5 5-μm (150 × 4.0 mm) and the MCH-N-cap-5 (monomeric octadecylsilane that is end-capped with a trimethylsilyl group) 5-μm (150 × 4.0 mm) packed columns are obtained from Varian Instruments, Palo Alto, CA, U.S.A.

Detector 1 is an Hitachi Model 100-30 with an Altex flow cell module Model 155 obtained as a total unit from Altex Scientific. The wavelength setting is 323 nm and the absorbance units full scale (a.u.f.s.) is 0.05. Detector 2 is a Waters Model 440 absorbance detector with a fixed wavelength of 313 nm; the a.u.f.s. is 0.02.

The mobile phase is a mixture of 1 m*M* pH 4.0 potassium phosphate—acetonitrile (93:7, v/v). The flow-rate is 1.5 ml/min. The mobile phase is degassed by bubbling helium through it for 10 min at a moderate rate.

A volume greater than 20 μl is injected to fill the sample loop adequately.

Protein measurements

Protein concentration of the treated sample supernatant is measured using the Lowry phenol method [7]. Reagent blanks were run for each organic solvent used. Absorbance readings were made at 750 nm, with a Model 200 spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.).

Sample preparation

Heparinized plasma or serum is collected in glass containers. All specimens are immediately frozen at −20°C unless the analysis can be done within 6 h. Plastic tubes are not used because of binding reactions between the nitroimidazoles and the plastic surface [8].

A 200-μl volume of standard, control or patient sample is placed into 12 × 75 mm glass test tubes. A 600-μl volume of protein-precipitating solvent consisting of methanol—acetonitrile (3:1) (alternatively 600 μl of ethanol or methanol or acetonitrile are used to establish this method) is added to each sample tube. Tubes are then vortexed for 10 sec, 200 μl of internal standard are added to each tube and then each tube is vortexed for 5 sec at medium speed. The tubes are allowed to stand at room temperature for 15 min to allow precipitation of the proteins to be completed. The tubes are centrifuged for 8 min at 1700 *g*.

RESULTS

Fig. 2 illustrates the effect of methanol, ethanol, acetonitrile and methanol—acetonitrile (3:1), at dilutions of 1/1 (serum to solvent ratio) to 1/10, on the amount of protein remaining in the serum sample. The three solvents and the solvent combination, where 1 volume of serum containing a known concentration of the two drugs was treated with 3 volumes of each particular solvent, were evaluated using the chromatographic system described with an Ultrasphere ODS 5-μm column. The ethanol-treated serum samples gave chromato-

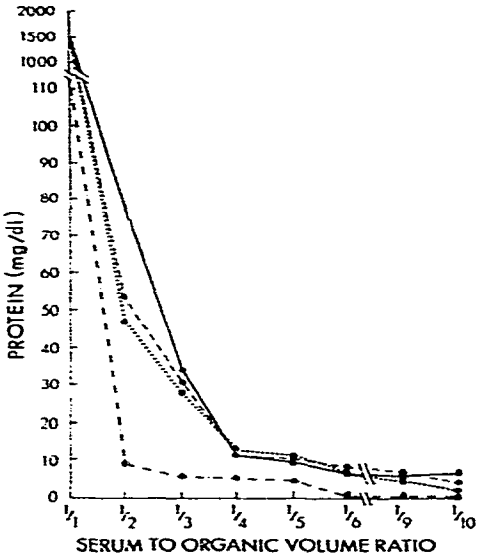
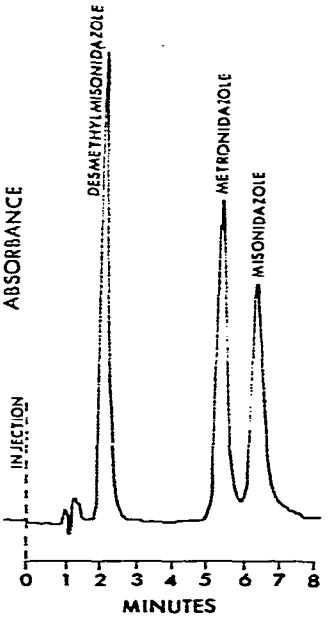


Fig. 2. Graph of the effect of the three organic solvents and a mixture of two of the solvents on the amount of protein remaining in a serum sample. ——, Methanol; – – –, ethanol; · · · ·, methanol—acetonitrile (3:1); —·—·—, acetonitrile.



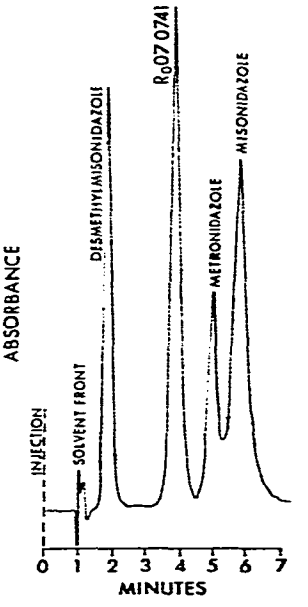


Fig. 3. Chromatogram of the three principle nitroimidazoles separated on an Ultrasphere ODS 5-μm column after serum sample preparation with methanol—acetonitrile (3:1).

Fig. 4. Chromatogram of the principle nitroimidazoles and of Ro 07-0741 on an Ultrasphere ODS 5-μm column.

TABLE I

PRECISION (CV%)

	Conc. (μg/ml)	Within day (n=6)		Day-to-day (n=10)*	
		313 nm	323 nm	313 nm	323 nm
Desmethylmisonidazole	7.5	2.9	1.2	7.3	5.7
	15.0	1.2	1.2	3.8	3.4
	60.0	0.9	2.4	2.2	2.1
Misonidazole	7.5	3.7	1.5	9.2	8.7
	15.0	3.2	2.2	5.7	5.3
	60.0	2.7	1.2	5.5	5.0

*Assayed in triplicate.

TABLE II

RETENTION TIMES

For operating conditions see *Chromatography* section.

Compound	Retention time (min)
Ro 11-6924	1.0
Desmethylmisonidazole	1.8
Ro 07-0741	3.7
Metronidazole	4.7
Misonidazole	5.4
Ro 07-0269	9.5
Ro 07-0913	13.0
Ro 07-0207	25.0

TABLE III

MISONIDAZOLE DOSAGE AND SERUM LEVEL CORRELATIONS

Dose (g/m^2)	n	Serum level (μg/ml)	
		Misonidazole	Desmethylmisonidazole
0.4	5	15.6 ± 2.6	2.0 ± 0.3
1.0	2	27.5 ± 0.5	10.0 ± 0.8
2.5	5	66.7 ± 0.9	13.3 ± 0.7

graphic results with a double peak for desmethylmisonidazole and a front shoulder on the misonidazole peak. Acetonitrile-treated serum samples chromatographed with the major portion of desmethylmisonidazole, co-eluting with the solvent front and a misonidazole peak that had a distorted front side. The methanol-treated serum sample gave chromatographic results with a small diffuse shoulder on the front side of the desmethylmisonidazole peak. When methanol—acetonitrile (3:1) was used as the solvent for serum sample preparation the separations were not interfered with by the injected sample. This is illustrated in Fig. 3, with metronidazole being added to verify the resolution of all three compounds.

The MCH-10 packing gives diffuse broad peaks that are not satisfactory. The MCH-5 packing gives clear resolution of all three compounds in 7 min. However,

the serum sample must be pretreated with methanol alone. The MCH-N-cap-5 packing can accomplish the separation of the three compounds in 4 min. Methanol must be used for the sample clean-up. The mobile phase for all the MCH packing materials is essentially the same as that used for the Ultrasphere ODS 5-μm packing.

Concentration linearity and precision using methanol—acetonitrile (3:1) and the Ultrasphere ODS 5-μm column gives a linear relationship from 0 to 120 μg/ml for desmethylmisonidzole with a CV of 2.4% ($y = 1.001x - 0.1$). Misonidazole is linear from 0 to 240 μg/ml with a CV of 1.2% ($y = 1.003x - 0.2$). Linearity above these values was not verified. The CV for within-day and day-to-day precision for the two wavelengths is shown in Table I. Recovery from serum or plasma is between 98% and 100% for both compounds and the internal standard. The detection limit at two times the signal-to-noise ratio using 200 μl of serum is 0.2 μg/ml for desmethylmisonidazole and 0.4 μg/ml for misonidazole. This represents an on-column injection of picogram amounts.

Fig. 4 shows that Ro 07-0741 elutes at 3.8 min, between desmethylmisonidazole and metronidazole with baseline resolution. Table II lists retention times for eight nitroimidazoles.

The serum levels of desmethylmisonidazole and misonidazole in adults given misonidazole on a g/m^2 basis are shown for three different dose levels in Table III.

DISCUSSION

Distortion of the chromatographic patterns by the small amount of organic solvent in the injection of the sample on to the column is not surprising. However, we have not been faced with this difficulty when we used 10-μm sized packings. Our utilization of 5-μm packings showed us that indeed a great deal of care must be used in the sample solvent to allow for precise chromatographic results. The work of Johnson et al. [9] described some related situations where precautions were indicated concerning the percentage of organic modifiers that could be injected containing the sample over and above which chromatographic distortions were likely to occur with ODS columns. Tseng and Rogers [10] were able to produce two sharp peaks or a sharp peak with a shoulder from a single pure compound that was being chromatographed by reversed-phase methods depending on the solvent combination used for the mobile phase and that which was used to introduce the sample onto the column.

Methanol—acetonitrile (3:1) used as the precipitating and sample solvating agent provides for very satisfactory chromatographic resolution with Ultrasphere ODS 5-μm columns. Methanol as the sample treatment agent provides the best results with the MCH-5 columns.

To avoid the large sample dilution and its accompanying loss of sensitivity of drug measurements, which Workman et al. [5] and Meering and Maes [6] utilize in their 1/9 dilution of serum with methanol, we use a 1/3 dilution of serum with methanol—acetonitrile (3:1). The presence of acetonitrile clears the supernatant whereas methanol alone leaves the supernatant slightly cloudy. We use one-fifth the amount of serum or plasma and we inject one-fifth the amount of the prepared sample onto the column as compared to Meering and Maes [6].

This procedure puts less protein onto the column per injection than these authors by 0.7 mg% (see Fig. 2). We routinely store our chromatography columns in acetonitrile which further removes any protein left on the column from this assay.

With this method we can use either 313 nm at 0.02 a.u.f.s. or 323 nm at 0.05 a.u.f.s. Table I lists the precision at each wavelength.

To our knowledge no one has published serum results for these compounds determined by HPLC from specific doses given to adult patients. The ratio between desmethylmisonidazole and misonidazole varies considerably between patients but is fairly constant for a given patient at a fixed sampling time from day to day.

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REFERENCES

1 G.E. Adams, J.F. Fowler and P. Workman, Brit. J. Cancer, 37 (Suppl. 3) (1978).
2 M.C. Walker and T.A. Strike, Cancer Clin. Trials, 3 (1980) 105.
3 R.C. Miller and E.J. Hall, Cancer Clin. Trials, 3 (1980) 85.
4 R.A. Marques, B. Stafford, N. Flynn and W. Sadée, J. Chromatogr., 146 (1978) 163.
5 P. Workman, C.J. Little, T.R. Marten, A.D. Dale, R.J. Ruane, I.R. Flockhart and M.M. Bleehen, J. Chromatogr., 145 (1978) 507.
6 P.G. Meering and R.A.A. Maes, J. Chromatogr., 225 (1981) 407.
7 W.H. Daugaaday, O.H. Lowry, N.J. Rosebrough and W.S. Fields, J. Lab. Clin. Med., 39 (1952) 663.
8 S. Riegleman, personal communication, 1980.
9 E.L. Johnson, R. Gloor and R.E. Majors, J. Chromatogr., 149 (1978) 571.
10 P.K. Tseng and L.B. Rogers, J. Chromatogr. Sci., 16 (1978) 436.