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Note

Estimation of the hypoxic cell-sensitiser misonidazole and its O-demethylated metabolite in biological materials by reversed-phase high-performance liquid chromatography

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It is widely held that the failure of radiotherapy to cure many human tumours is due in part to the presence of poorly oxygenated malignant cells which are comparatively resistant to radiation [1, 2]. Considerable interest has therefore been shown in hypoxic cell-sensitisers [3] of which one of the most promising is the 2-nitroimidazole, misonidazole (Roche) (for review see ref. 3). Preliminary clinical trials with this compound have demonstrated radiosensitisation in man [4] and it is now under investigation at a number of radiotherapy centres.

As both the radiosensitising effect and the toxicity of misonidazole are dose-dependent [5], a knowledge of the pharmacokinetics of the drug in individual patients is particularly desirable. Five techniques for the assay of misonidazole have been described previously. Reported UV spectrophotometric [6] and

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polarographic [7] techniques have the disadvantage that they determine total nitroimidazole concentration, failing to discriminate between misonidazole and its O-demethylated metabolite (Ro 05-9963). This separation has been achieved by paper chromatography [8], thin-layer chromatography (TLC) [9] and gas—liquid chromatography (GLC) [9]. However, the two former methods do not lend themselves to accurate routine analysis and the latter method requires a time-consuming extraction and derivatisation procedure.

It has been shown that C_{18}-bonded silica is the best of the commercially available bonded supports for the reversed-phase separation of misonidazole and its O-demethylated metabolite [10]. More recent work has, however, indicated that even better resolution can be achieved on a C_{22}-bonded silica [11].

The present paper describes a rapid reversed-phase high-performance liquid chromatographic (HPLC) method for the assay of misonidazole and its O-demethylated metabolite in biological materials.

EXPERIMENTAL

Essentially the same method has been employed in four separate laboratories, each using different HPLC equipment. To avoid repetition, the present communication gives precise details of the technique used in one laboratory.

Reagents
The following nitroimidazoles were synthesised and supplied by Roche Products (Welwyn Garden City, Herts., Great Britain): 1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol (misonidazole, Ro 07-0582) (I); 1-(2-nitroimidazol-1-yl)-2,3-propandiol (Ro 05-9963) (II); 1-(2-nitroimidazol-1-yl)-3-chloropropan-2-ol (Ro 07-0269) (III); and 1-(2-nitroimidazol-1-yl)-3-ethoxypropan-2-ol (Ro 07-0913) (IV). The structural formulae of these compounds are shown in Fig. 1.

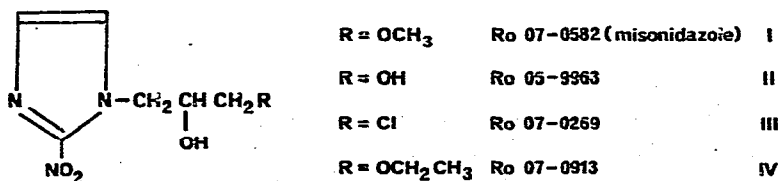|	
R = OCH$_3$	Ro 07-0582 (misonidazole) I
R = OH	Ro 05-9963 II
R = Cl	Ro 07-0269 III
R = OCH$_2$CH$_3$	Ro 07-0913 IV

Fig. 1. Structural formulae of nitroimidazoles.

HPLC-grade methanol was obtained from Rathburn Chemicals (Walkerburn, Great Britain). Water was twice-distilled in glass and both solvents were passed through appropriate 0.45 μm Millipore filters and de-aerated under vacuum before use.

Sample preparation
The following procedure was found to be suitable for samples of urine, tissue homogenate (10—20%, w/v) and heparinised blood plasma. The sample was treated with 9 vol. methanol containing the internal standard Ro 07-0269 (11.1 μg/ml) and mixed thoroughly. After centrifugation (2000 g, 10 min) the super-

natant was removed for HPLC analysis. Alternatively, the plasma was deproteinated by passage through Amicon CF50A filter cones at 1000 g (60 min). The ultrafiltrate was then diluted with methanol to the concentration of the eluant prior to chromatography. Standards, prepared by adding known amounts of misonidazole and Ro 05-9963 in methanol to plasma collected before drug administration, were analysed as for experimental samples.

Chromatography

Samples (10 μl) of the methanolic extracts were chromatographed at ambient temperature using a Waters Model ALC/GPC-244 liquid chromatograph equipped with a U6K sample loop injector and a μBondapak C_{18} (octadecylsilane) column (30 cm × 3.9 mm I.D., particle size = 10 μm) (Waters Assoc., Milford, Mass., U.S.A.). The mobile phase, consisting of 19% methanol—water, was delivered at a constant flow-rate of 2 ml/min (pressure = 2000 p.s.i.). The column effluent was monitored at 313 nm using a Waters Model 440 (absorbance detector coupled to a Servoscribe chart recorder. Peak areas were determined using a Varian CDS 111 computer.

RESULTS

Fig. 2a shows a typical chromatogram of a methanol extract of blood plasma taken from a cerebral tumour patient (CA) immediately before administration of misonidazole. The internal standard was deliberately omitted. The chromatogram illustrated in Fig. 2b is of a methanol extract of the same plasma spiked with misonidazole (peak 2) and Ro 05-9963 (peak 1) and containing the internal standard Ro 07-0269 (peak 3). Ro 05-9963, misonidazole and Ro 07-0269 had retention times of 2.3 min, 4.1 min and 4.8 min respectively. The three peaks were completely resolved from each other and from material eluting at the solvent front. Typical height equivalents to a theoretical plate for misonidazole, Ro 05-9963 and Ro 07-0269 were respectively 0.15 mm, 0.25 mm and 0.15 mm.

Comparison of Fig. 2a and b shows that the blank plasma contained no components capable of interfering with peaks 1—3. Similar results were obtained for extracts of control urine and tissue homogenate.

Fig. 2c shows a chromatogram of the methanol extract of plasma taken from the same patient 2 h after the administration of misonidazole (3 g/m²). It may be seen that the chromatogram contains peaks corresponding to misonidazole and Ro 05-9963.

The efficiency of recovery of misonidazole, Ro 05-9963 and Ro 07-0269 from biological media after protein precipitation was always >95%. Plots of normalised peak area ratio (peak area:peak area internal standard) against concentration were linear over the concentration ranges studied (2—1000 μg/ml misonidazole; 2—50 μg/ml Ro 05-9963) and had zero intercepts. Plots of peak height ratio were also linear. The coefficient of variation calculated for ten replicate analyses (peak height ratio) was 2.9% for misonidazole and 1.9% for Ro 05-9963.

Allowing a minimum signal-to-noise ratio of two, the lower limit of sensitivity of the protein precipitation method was approximately 5 μg/ml sample
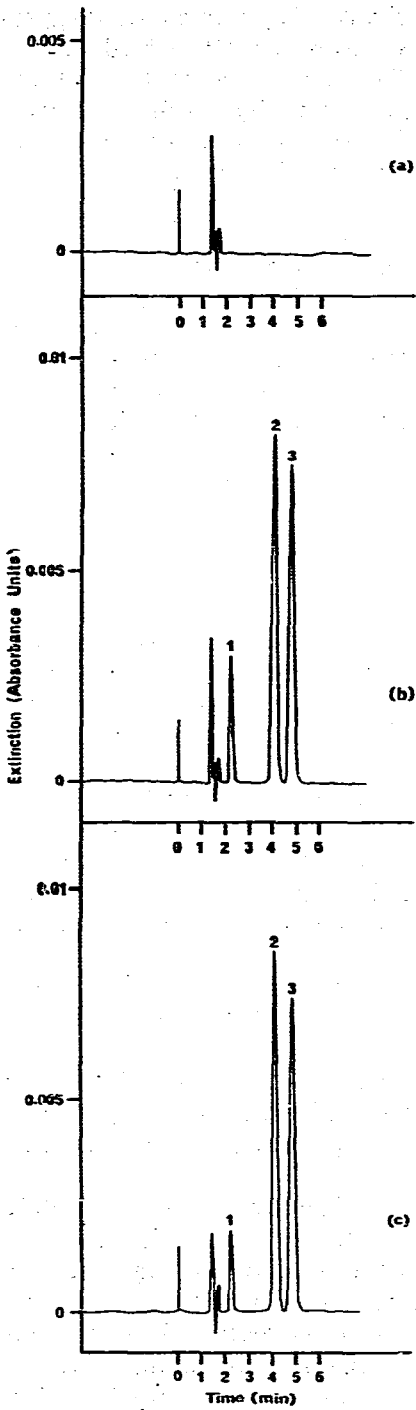
(plasma, urine or tissue homogenate) for misonidazole and 2 μg/ml for the O-demethylated metabolite. This represents an on-column injection of 5 ng and 2 ng respectively.

In some studies Ro 07-0913 was used as the internal standard. This compound had a retention time of 7.3 min and was completely resolved from the other nitroimidazoles. To check the precision of the HPLC method some test samples were analysed by both HPLC and GLC [9]. Good agreement was observed for both misonidazole and Ro 05-9963.

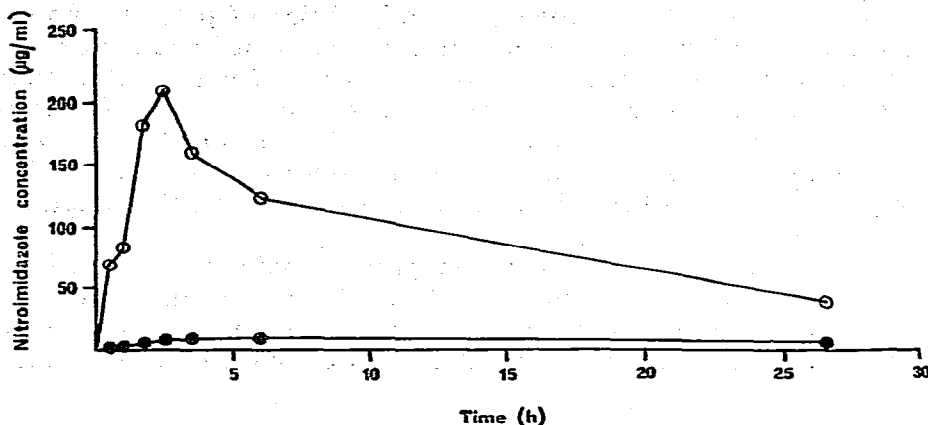An application of the present HPLC technique is demonstrated in Fig. 3

Fig. 3. Plasma time—course of misonidazole (o) and Ro 05-9963 (•) for a patient with a cerebral glioma after administration of 3 g/m² misonidazole orally. This patient exhibited abnormally high plasma levels of misonidazole.

which shows the plasma time-course of misonidazole and Ro 05-9963 for a cerebral tumour patient (EI) receiving 3 g/m² misonidazole orally. This patient exhibited abnormally high plasma levels of misonidazole, thus illustrating the importance of drug monitoring. It may also be seen that the O-demethylated metabolite constitutes a small, but not insignificant, proportion of the total plasma nitroimidazole concentration.

DISCUSSION

The rapid HPLC technique described in the present paper has several advantages. Firstly, unlike previous spectrophotometric [6] and polarographic [7] techniques, the method is specific for both misonidazole and its O-demethylated metabolite Ro 05-9963. Secondly, unlike the reported GLC technique [9]

Fig. 2. HPLC of methanol extracts of blood plasma from a patient (CA) with a cerebral glioma. Chromatographic conditions: column, μ Bondapak C_{18} (20 cm × 3.9 mm I.D.; particle size = 10 μm); mobile phase, methanol—water (19:81); flow-rate, 2ml/min; column pressure, 2000 p.s.i.; temperature, ambient; detection, absorbance at 313 nm; sample volume, 10 μl. (a) Sample taken immediately before administration of misonidazole. Internal standard was omitted. (b) Same sample as (a) but spiked with Ro 05-9963 (peak 1, 20 μg/ml plasma) and misonidazole (peak 2, 100 μg/ml plasma). Peak 3 corresponds to the internal standard (11.1 μg/ml methanol). (c) Sample taken 2 h after administration of 3 g/m² misonidazole orally.

it requires only one simple protein precipitation step and no derivatisation prior to analysis. Thirdly, unlike the previous paper chromatography and TLC methods [8, 9], it lends itself readily to accurate quantitation on a routine basis. Finally, the sensitivity of the method is comparable with, or better than, these alternative techniques. This is adequate for the assay of misonidazole and Ro 05-9963 following the administration of therapeutic doses of misonidazole. Furthermore, the sensitivity of the method could be improved by concentration of the methanol extract or by using the Amicon filter cones.

The HPLC method is currently being employed to determine the levels of misonidazole and Ro 05-9963 in tissues and body fluids of patients and experimental animals. Digestion with β-glucuronidase has also allowed the estimation of glucuronide derivatives of these compounds in urine. The present method does not, however, allow the estimation of the nitro-reduction product 1-(2-aminoimidazol-1-yl)-3-methoxypropan-2-ol (Ro 07-7273) which has been identified as a metabolite [8, 9]. This compound does not possess the UV absorption properties of the nitroimidazoles and we are currently evaluating various alternative methods for its detection.

The HPLC method described here has also proved useful for the assay of various other nitroimidazoles including the 5-nitroimidazole, metronidazole (2-(2-methyl-5-nitro-1-imidazolyl)-ethanol, Flagyl, May and Baker), used clinically both as an anti-trichomonal agent and as a hypoxic cell-sensitiser.

REFERENCES

1 L.H. Gray, A.D. Conger, M. Ebert, S. Hornsey and O.C. Scott, Brit. J. Radiol., 26 (1953) 638.
2 R.H. Thomlinson and L.H. Gray, Brit. J. Cancer, 9 (1955) 539.
3 J.F. Fowler, G.E. Adams and J. Denekamp, Cancer Treatment Rev., 3 (1976) 227.
4 R.H. Thomlinson, S. Dische, A.J. Gray and L.M. Errington, Clin. Radiol., 27 (1976) 167.
5 S. Dische, M.I. Saunders, M.E. Lee, G.E. Adams and I.R. Flockhart, Brit. J. Cancer, 35 (1977) 567.
6 R. Johnson, C. Gomer and J. Pearce, Int. J. Radiol. Oncol. Biol. Phys., 1 (1976) 593.
7 J.L. Foster, I.R. Flockhart, S. Dische, A. Gray, I. Lenox-Smith and C.E. Smithen, Brit. J. Cancer, 31 (1975) 679.
8 A.J. Varghese, S. Gulyas and J.K. Mohindra, Cancer Res., 36 (1976) 3761.
9 I.R. Flockhart, P. Large, S.L. Malcolm, T.R. Marten and D. Troup, Xenobiotica, in press.
10 C.J. Little, A.D. Dale and M.B. Evans, J. Chromatogr., in press.
11 C.J. Little, A.D. Dale and M.B. Evans, J. Chromatogr., in press.