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Note

Gradient high-performance liquid chromatographic method for simultaneous assay of the radiosensitizers etanidazole (SR 2508) and pimonidazole (Ro 03-8799) in biological materials

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Following successful Phase I studies [1–3], the 2-nitroimidazoles etanidazole and pimonidazole are currently under individual evaluation in Phase II–III trials as potential radiosensitizers in the treatment of human cancer. Because their dose-limiting side-effects are different, a Phase I study of these agents administered together is now in progress [4, 5].

In single agent studies, pimonidazole and its N-oxide metabolite are analysed by an isocratic reversed-phase paired-ion high-performance liquid chromatographic (HPLC) technique [6], while etanidazole is determined by a modification [7] of the isocratic reversed-phase HPLC method for misonidazole [8]. We now report a simple and rapid gradient HPLC technique for the simultaneous analysis of all three compounds in biological material.

EXPERIMENTAL

Materials

Etanidazole (I) was supplied by SRI International (Stanford, CA, U.S.A.) and the U.S. National Cancer Institute (Bethesda, MA, U.S.A.). Misonidazole (II), pimonidazole (III) and its N-oxide metabolite (IV) were supplied by Roche Products (Welwyn Garden City, U.K.). Structures are shown in Fig. 1.

Acetonitrile and methanol (both HPLC grade) were obtained from Rathburn Chemicals (Walkerburn, U.K.). 1-Heptanesulphonic acid (HSA, sodium salt) was obtained from Fisons (Loughborough, U.K.). Water was distilled once and then deionised using a Milli-Q purification system (Millipore, Molsheim, France).

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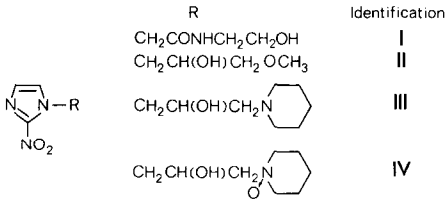

Fig. 1. Structures of etanidazole [I, N-(2-hydroxyethyl)-2-nitroimidazolyl acetamide (SR 2508)], pimonidazole {III, α-[(2-nitro-1-imidazolyl)methyl]-1-piperidine ethanol hydrochloride (Ro 03-8799)}, pimonidazole N-oxide {IV, α-[(2-nitro-1-imidazolyl)methyl]-1-piperidine ethanol-1-oxide (Ro 31-0313)} and misonidazole [II, 1-(2-nitroimidazole-1-yl)-3-methoxypropan-2-ol (Ro 07-0582)].

Mobile phases were passed through 0.45-μm Millipore filters and degassed under vacuum before use.

Sample preparation

The following procedure was used for heparinised blood plasma and tissue homogenates [16–33% (w/v), depending on biopsy size]. Samples (200 μl) were deproteinised by the addition of 1 ml acetonitrile containing misonidazole (2 mg l^{-1}) as internal standard, and mixed thoroughly. After centrifugation (1600 *g*, 15 min at 4°C) the supernatant was removed and dried under vacuum using a Savant Speed Vac sample concentrator coupled to a Model RT 100-A refrigerated condensation trap (Savant, Farmingdale, NY, U.S.A.). Residues were resuspended in 100 μl of 7% methanol mobile phase (see later) and aliquots (15–25 μl) were injected for analysis. Standards containing known concentrations of the three compounds were prepared by spiking into pooled human plasma or foetal calf serum (Sera-Lab, Crawley Down, U.K.) and processed simultaneously with test samples.

Chromatography

Chromatography was carried out using equipment and columns supplied by Waters Assoc. (Milford, MA, U.S.A.). This comprised two Model 6000A pumps; a Model 710B WISP autoinjector; a Model 660 solvent programmer; a Z-module containing a μBondapak C_{18} Rad-Pak cartridge column (10 cm×8 mm I.D.; 10-μm irregular particles, 10% carbon load, end-capped), protected by a μBondapak C_{18} Guard-Pak precolumn; and a Model 440 fixed-wavelength UV detector set at 313 nm. The latter was connected to a two-channel chart recorder (Servoscribe, Smiths Industries, Cricklewood, London U.K.). Mobile phase A consisted of 7% methanol in 0.2 *M* glycine–HCl buffer, pH 2.5, containing 5 m*M* HSA, and mobile phase B of 40% methanol in the same buffer. A linear gradient was run over 8 min starting at 90% A, 10% B, and finishing at 0% A, 100% B, using a flow-rate of 4 ml min^{-1}. Initial conditions were restored immediately after the completion of the gradient, and the total reequilibration time preceding the next injection was 3.5 min. Chromatography was carried out at room temperature. Quantitation was by peak-height ratio with reference to standards spiked in a volume of 5 μl methanol per 100 μl plasma. Concentrations of pimonidazole are reported as the hydrochloride salt.

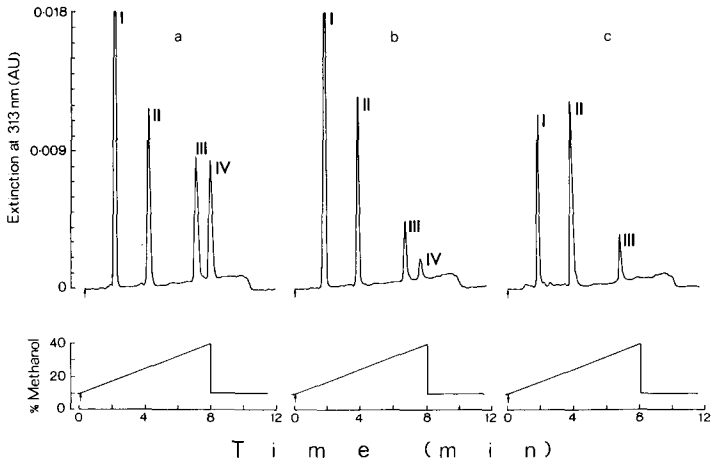

Fig. 2. Chromatograms of resuspended dried acetonitrile extracts. In all cases the internal standard misonidazole was included in the acetonitrile used for extraction, at a concentration of 2 mg l^{-1}. (a) Foetal calf serum spiked with etanidazole, pimonidazole and its N-oxide metabolite (all at 13 μg ml^{-1} of serum). (b) Patient's plasma taken 30 min after intravenous administration of 0.56 g m^{-2} of both etanidazole and pimonidazole; the plasma contained 29 μg ml^{-1} etanidazole, 6 μg ml^{-1} pimonidazole and 2 μg ml^{-1} N-oxide metabolite. (c) Aqueous homogenate (33%, w/v) of brain tumour biopsied from the same patient, also at 30 min; the homogenate contained 6 μg ml^{-1} etanidazole and 5 μg ml^{-1} pimonidazole, while the N-oxide metabolite was below the limit of detection (0.3 μg ml^{-1}). Chromatographic conditions: column, μBondapak C_{18} Rad-Pak (10 cm×8 mm I.D.; 10 μm particle size); mobile phase, linear gradient from 10.3 to 40% methanol in 0.2 *M* glycine-HCl buffer, pH 2.5, containing 5 m*M* HSA; flow-rate, 4 ml min^{-1}; temperature, ambient; column pressure, 7 MPa; detection, absorbance at 313 nm; sample volume, 15 μl; chart speed, 10 mm min^{-1}. Peaks: I = etanidazole; II = misonidazole (internal standard); III = pimonidazole; IV = N-oxide metabolite of pimonidazole.

RESULTS

Fig. 2a shows a chromatogram of a resuspended, dried acetonitrile extract of foetal calf serum spiked with etanidazole (I), pimonidazole (III), its N-oxide metabolite (IV), and containing the internal standard, misonidazole (II). Retention times were 1.8, 7.0, 8.0 and 4.1 min, respectively. Identical results were obtained with spiked pooled human plasma and foetal calf serum, the latter being used routinely for convenience and safety. No interfering peaks were detected in control plasma or serum. Fig. 2b shows a typical chromatogram of an extract of plasma from a patient who had received 0.56 g m^{-2} of both etanidazole and pimonidazole, 30 min previously. Fig. 2c shows a chromatogram of a resuspended, dried acetonitrile extract of a homogenate of brain tumour, biopsied from the same patient also at 30 min. Etanidazole, pimonidazole and its N-oxide metabolite are readily identifiable in plasma whilst the first two only are clearly visible in the tumour.

Using this method it can be seen that all four nitroimidazoles are readily resolved from each other. The efficiency of recovery from plasma or serum was $\geq$88% in all cases. Plots of peak-height ratio (analyte/standard) against concentration were linear over the pharmacologically relevant ranges studied (8–250 μg ml^{-1}

TABLE I

COEFFICIENT OF VARIATION AND ACCURACY FOR ANALYSIS OF FOETAL CALF SERUM SPIKED WITH ETANIDAZOLE, PIMONIDAZOLE AND PIMONIDAZOLE N-OXIDE

Compound	Concentration spiked ($\mu g\ ml^{-1}$)	Coefficient of variation* (%)	Accuracy** (%)
Etanidazole	100	1.0	94
	1	11.1	113
Pimonidazole	20	1.0	102
	1	9.0	104
Pimonidazole N-oxide	5	1.8	99
	1	11.2	105

*For ten replicate analyses.

**For analysis of known quality control samples using the standard assay system, where four to six standards are included with each analysis of ten to twenty duplicate unknowns. Results are the mean of four replicate samples, each analysed in duplicate.

etanidazole, 2–90 $\mu g\ ml^{-1}$ pimonidazole and 0.5–15 $\mu g\ ml^{-1}$ N-oxide metabolite) and all exhibited zero intercepts. The within-day coefficients of variation for the assay of pharmacological concentrations were very acceptable, though increasing at lower concentrations (Table I). The accuracies ranged from 94 to 113%, with an overall mean of 103%.

Allowing a minimum signal-to-noise ratio of 2, the lower limits of detection were 0.4, 0.5 and 0.3 $\mu g\ ml^{-1}$ for etanidazole, pimonidazole and N-oxide metabolite, respectively, using an injection volume of 25 μl. These represent on-column quantities of 10, 12.5 and 7.5 ng, respectively. Forty-eight prepared samples can be chromatographed in 9 h 12 min. With routine daily washing, and changes of guard column approximately every 100 samples, the analytical column used has shown no deterioration in performance after analysis of 380 serum, plasma and tissue extracts over a period of two months.

DISCUSSION

The gradient HPLC method described here allows the simultaneous analysis of the radiosensitizers etanidazole and pimonidazole, together with the N-oxide metabolite of the latter, in biological materials. Compared to previous isocratic techniques [6–8] the present method has the advantage of a reduced overall analysis time, a requirement for only one internal standard and a more economic use of available sample resources. A gradient method developed independently at the same time elsewhere [9] required a longer run-time, and in addition gave less symmetrical peak shapes, probably as a result of the omission of the paired-ion reagent from the low organic phase component of the binary gradient system.

The method described here is now being used to study the rodent and human pharmacokinetics of etanidazole and pimonidazole, the combination of which shows considerable promise for the radiosensitization of human tumours.

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