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Note

Reversed-phase high-performance liquid chromatographic method for the simultaneous determination of the 2-nitroimidazole benznidazole and its amine metabolite in biological materials

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Nitroimidazoles are widely used in medicine to treat a variety of microbial infections and as hypoxic-cell sensitizers in the radiotherapy and chemotherapy of cancer. The 2-nitroimidazole misonidazole has been extensively evaluated in the clinic as a radiosensitizer, but full radiosensitization has not been achieved through dose-limiting neurotoxicity [1]. It has been proposed that the cytotoxicity and neurotoxicity of nitroimidazoles are associated with the reduction of the nitro group to its corresponding amine, possibly as a result of the production of highly reactive nitroso, hydroxylamine or other intermediates [2, 3].

(a) (b)

Fig 1 Structures of (a) benznidazole and (b) benznidazole amine

Benznidazole [N-benzyl-(2-nitro-1-imidazoyl)acetamide, Fig. 1) is a 2-nitroimidazole analogue of misonidazole used mainly in the treatment of American Trypanosomiasis (Chagas' disease) [4]. On the basis of studies in our unit [5] benznidazole is currently being evaluated in a national Medical Research Council trial as a chemosensitizer in combination with the cytotoxic nitrosourea 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) for the treatment of brain tumours. A number of mechanisms have been proposed for nitro-

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imidazole-mediated chemosensitisation including alterations in drug pharmacokinetics [6] and/or mechanisms operating at the cellular level through nitroreduction of the nitroimidazole [7, 8].

Previous analytical techniques for benznidazole have employed differential-pulse polarography [9] and column liquid chromatography (LC) [10], but neither technique was capable of detecting reduced metabolites. In view of the putative role of reductive metabolism in nitroimidazole toxicity and chemosensitisation, we have developed a novel, rapid, paired-ion LC method for the simultaneous assay of benznidazole and its corresponding amine in biological fluids and tissues

EXPERIMENTAL

Materials

N-Benzyl-(2-nitro-1-imidazoyl)acetamide (Ro 07-1051) was supplied by Roche Products (Welwyn Garden City, U K) as was 1-(2-nitroimidazoyl-1-yl)-3-*n*-butoxypropan-2-ol (Ro 07-0602) used as the internal standard. N-Benzyl-(2-amino-1-imidazoyl)acetamide (Ro 11-1721), benznidazole amine, was provided as the hydrochloric salt by Roche (Basle, Switzerland).

Acetonitrile (HPLC S-grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.) Octanesulphonic acid (HPLC grade) was supplied as the sodium salt by Fisons Scientific Apparatus (Loughborough, U K.). Water was distilled once and then deionised by passing it through a four-cartridge Milli-Q water purification system (Millipore, Molsheim, France) The mobile phase was passed through the appropriate 0.45-μm Millipore filters and thoroughly degassed under vacuum before use

Drug administration

Adult inbred male C3H/He and BALB/c mice were obtained from OLAC Southern (Bicester, U.K.) and our own breeding colony. They were allowed laboratory chow and water ad lib.

Benznidazole was supplied in powder form and was suspended in 50% polyethylene glycol (PEG, mol wt 400) in Hanks' buffered salt solution (HBSS) before intraperitoneal (i.p.) injection in a volume of 0.01 ml g^{-1} body weight, at a dose of 2 5 mmol kg^{-1} (650 mg kg^{-1}). All animals were sacrificed 3 h after i.p injection Whole blood was removed by cardiac puncture into heparinised syringes and centrifuged at 3000 *g* for 15 min to obtain plasma Tissues were rapidly removed and immediately snap-frozen on dry ice. For urinary recovery studies five to six mice were contained in a Urimax metabolism cage and urine was collected for 24 h on dry ice. All samples were handled at 4°C and stored at −20°C before analysis.

Sample preparation

The following procedure was found to be suitable for samples of urine, heparinised blood plasma and tissue homogenates (33%, w/v). Each sample was deproteinised by the addition of 2 vols. of methanol containing the internal standard (20 mg l^{-1} methanol) and mixed thoroughly. After centrifugation (3000 *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). The residues were resuspended in running buffer (100 μl) and an aliquot (20 μl) removed for LC analysis Standards were treated identically after spiking pooled human plasma with known concentrations of benznidazole and its amine metabolite in methanol

Chromatography

All chromatography was carried out using equipment and columns supplied by Waters Assoc (Milford, MA, U.S.A) Aliquots (20 μl) of the resuspended dried methanolic extract were chromatographed at ambient temperature using a Radial Compression separation system The equipment used included two Model 6000 A chromatography pumps and a Model 410B Waters Intelligent Sample Processor (WISP) which were connected to a Z-module The analyses were performed on a μBondapak C_{18} octadecylsilane Rad-Pak column (10 cm × 8 mm I D containing 10-μm particles). The mobile phase consisted of 25% acetonitrile in 0 2 *M* glycine—hydrochloric acid buffer, pH 2 5, containing 5 m*M* octanesulphonic acid, which was delivered at a constant flow-rate of 3.5 ml min^{-1}. The absorbance was monitored at 229 and 313 nm using a Model 441 and a Model 440 fixed-wavelength UV detector, respectively, which were connected to a twin-channel Servoscribe chart recorder (chart speed 10 mm min^{-1})

RESULTS AND DISCUSSION

Chromatography was initially attempted on several column packings, including cyanonitrile (CN), octylsilane (C_8), and octadecylsilane (C_{18}, spherical beads) but the separation and peak shapes were generally poor. However, good resolution and peak shape were obtained with μBondapak C_{18} Rad-Pak columns

Fig. 2a shows a chromatogram of a resuspended dried methanolic extract of plasma from a control mouse which received 50% PEG—HBSS i.p., the vehicle for benznidazole administration, and containing the internal standard, which is detected at both 229 and 313 nm (peak 3). Fig 2b shows a chromatogram of an extract of pooled C3H mouse plasma spiked with benznidazole amine (peak 1) and benznidazole (peak 2) and also containing the internal standard. Benznidazole is detectable at both wavelengths while the amine is only seen at 229 nm. Fig. 2c shows a typical chromatogram of an extract of plasma from a mouse which received 2 5 mmol kg^{-1} benznidazole i p. As well as the internal standard, there is a large peak which corresponds to benznidazole (peak 2) with a much smaller peak at the lower limit of detection corresponding to benznidazole amine (peak 1) Because of its greater versatility all the subsequent chromatograms will show only the peaks detected at 229 nm Benznidazole amine, benznidazole and internal standard had retention times of 4 2, 5 5 and 8.1 min, respectively, and typical values for the peak-height equivalents to a theoretical plate were 0.65, 0.61 and 0.65, respectively.

Fig. 3a and b show chromatograms of a resuspended dried methanolic extract of a 33% liver homogenate from a BALB/c mouse which had received

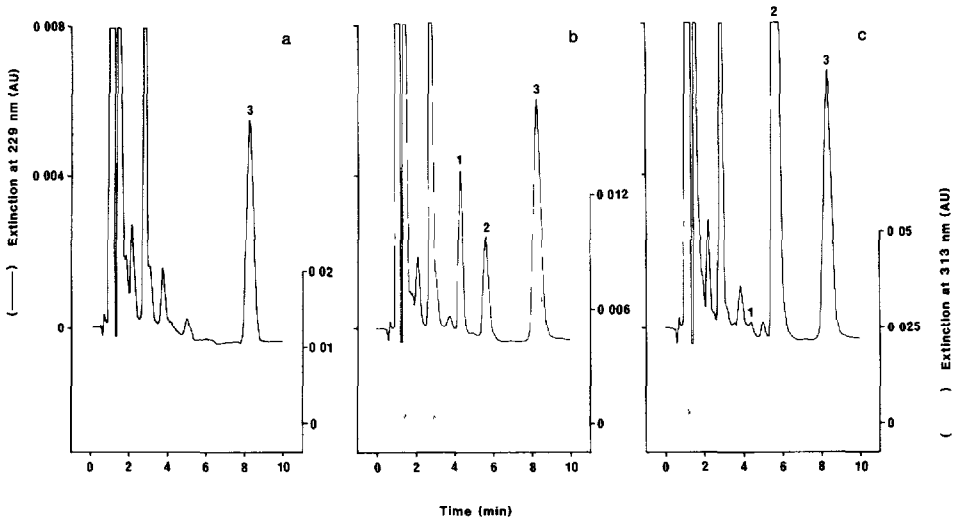

Fig 2 Chromatograms of resuspended dried methanolic extracts of blood plasma from female C3H mice (a) Sample taken from a C3H mouse 3 h after an i p dose of 50% PEG—HBSS, containing internal standard (20 mg l^{-1} in methanol) (b) Plasma spiked with benznidazole amine (10 μg ml^{-1} of plasma) and benznidazole (10 μg ml^{-1} of plasma), containing internal standard (c) Sample taken 3 h after administration of benznidazole (2 5 mmol kg^{-1} i p) containing benznidazole amine at the lower limit of detection (0 3 μg ml^{-1} of plasma), benznidazole (125 μg ml^{-1} of plasma) and internal standard Chromatographic conditions column, μBondapak C_{18} Rad-Pak, mobile phase, 25% acetonitrile in 0 2 *M* glycine—hydrochloric acid buffer, pH 2 5, containing 5 m*M* octanesulphonic acid, flow-rate, 3 5 ml min^{-1}, column pressure, 10 MPa, temperature, ambient, detection, absorbance at 229 and 313 nm, sample volume, 20 μl, chart speed, 10 mm min^{-1} Peaks 1 = benznidazole amine, 2 = benznidazole, 3 = internal standard

drug vehicle or benznidazole, respectively Fig 4a and b show chromatograms of a resuspended dried methanolic extract of pooled, 1 10 diluted, C3H mouse urine collected for 24 h after drug vehicle or benznidazole, respectively. The chromatograms of Fig. 3b and of Fig. 4b both contain peaks that correspond to benznidazole and its amine metabolite, the latter being present in similar amounts to the parent drug The control chromatograms for plasma, liver and urine (Figs 2a, 3a and 4a) contain no interfering peaks In all chromatograms internal standard, benznidazole and the amine metabolite were clearly resolved from each other The efficiency of recovery of benznidazole, its amine metabolite and internal standard from biological media after protein precipitation with methanol, drying and resuspension was always $\geqslant$ 90%. Plots of normalised peak-height ratios (analyte internal standard) against concentration were linear over the concentration ranges studied (0 5– 1000 μg ml^{-1} benznidazole and 0.5– 100 μg ml^{-1} benznidazole amine) and had zero intercepts The coefficient of variation calculated for eight replicate analyses (normalised peak-height ratio) was 1 7 and 3.2% for benznidazole amine and benznidazole, respectively as detected at 229 nm, and 3 4% for benznidazole as detected at 313 nm. Allowing a minimum signal-to-noise ratio of 2, the lower limit of sensitivity of this sample preparation technique was approximately 0 2—0 5 μg ml^{-1} for an injection volume of 20 μl. This represents an on-column injection of 5—10 ng

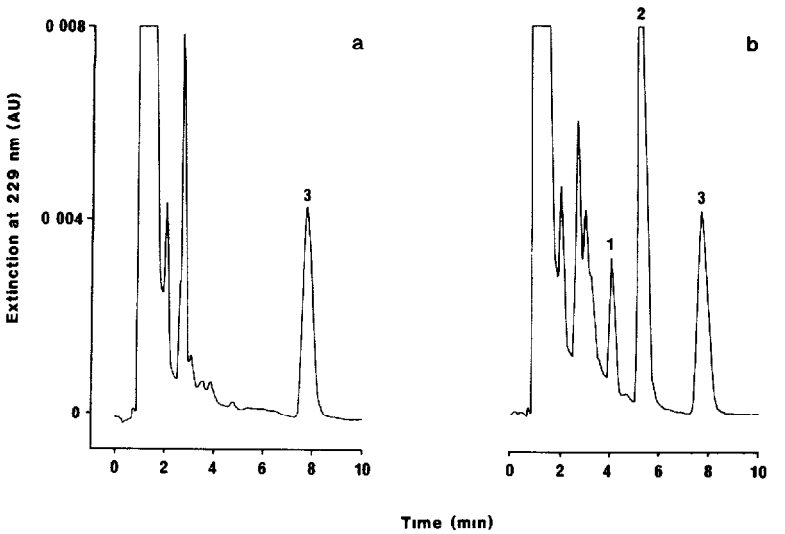

Fig 3 Chromatograms of resuspended dried methanolic extracts of 33% (w/v) liver homogenates from female BALB/c mice treated 3 h previously with (a) 50% PEG—HBSS i p and containing internal standard (20 mg l^{-1} in methanol) and (b) 2 5 mmol kg^{-1} benznidazole i p and containing benznidazole amine (12 7 $\mu g\ ml^{-1}$ of homogenate, 38 2 $\mu g\ g^{-1}$ of liver), benznidazole (60 3 $\mu g\ ml^{-1}$ of homogenate, 181 $\mu g\ g^{-1}$ of liver) and internal standard Chromatographic conditions were as for Fig 2 Peaks 1 = benznidazole amine, 2 = benznidazole, 3 = internal standard

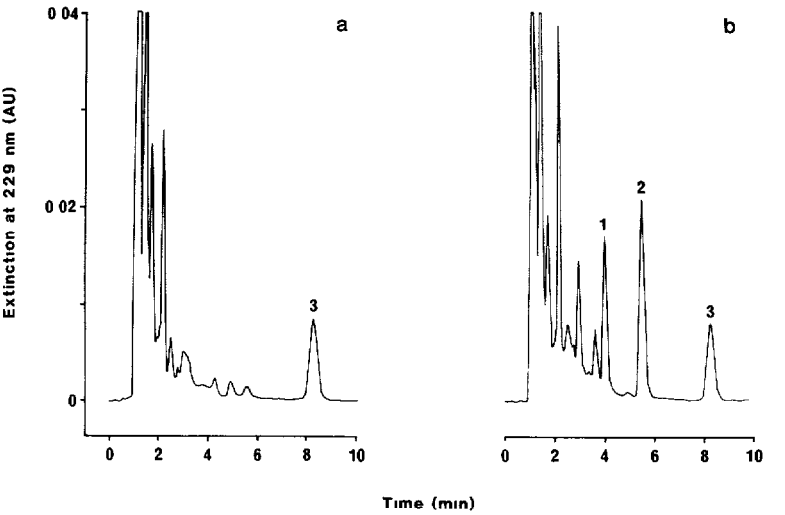

Fig 4 Chromatograms of an extract of 1 10 diluted 24-h urine collected from female C3H mice administered (a) 50% PEG—HBSS vehicle and containing internal standard (20 mg l^{-1} in methanol), and (b) 2 5 mmol kg^{-1} benznidazole i p containing benznidazole amine (33 4 $\mu g\ ml^{-1}$, 334 $\mu g\ ml^{-1}$ of urine), benznidazole (53 4 $\mu g\ ml^{-1}$, 534 $\mu g\ ml^{-1}$ of urine) and internal standard Chromatographic conditions were as for Fig 2 Peaks 1 = benznidazole amine, 2 = benznidazole, 3 = internal standard

This paired-ion LC technique has several advantages over previous assays for nitroimidazoles and their amine metabolites It is rapid and selective for both benznidazole and its amine metabolite at toxicological and pharmacological concentrations in biological fluids and tissues [10] There is a simple protein precipitation step, and no complicated derivatization process as was required for detection of the amine metabolite of misonidazole in a previous LC assay [11]. Unlike the thin-layer chromatographic [11] and paper chromatographic [12] assays for misonidazole and its amine, this technique does not require radiolabelled drug and readily lends itself to accurate quantitation of benznidazole and its amine metabolite on a routine basis The use of two wavelengths or sensitivities allows simultaneous monitoring of high concentrations of benznidazole and much lower concentrations of the amine metabolite, as occurs after administration of 2.5 mmol kg^{-1} benznidazole, an active dose in our chemosensitisation studies in mice [13].

Chromatograms of 24-h urine from mice administered benznidazole contained a number of smaller peaks eluting between the solvent front and the benznidazole amine peak which were not present in control urine. Although these peaks have not been identified they may be additional hydrophilic metabolites or metabonates of benznidazole.

It is interesting to note that there was practically no amine detected in the plasma, but very high concentrations detected in the liver, implicating this tissue as a major site of nitroreduction in BALB/c mice Very high concentrations of amine were also seen in the C3H mouse urine

This LC method is currently being employed to investigate the reductive metabolism of benznidazole in vivo in mice In addition, studies on the nitroreduction of benznidazole to its amine metabolite in humans are also in progress.

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REFERENCES

1 S Dische, M I Saunders, P Anderson, M R L Stratford and A M Minchinton, Int J Radiat Oncol Biol Phys , 8 (1982) 335
2 A M Rauth, Int J Radiat Oncol Biol Phys , 10 (1984) 1293
3 P J Eifel, D M Brown, W W Lee and J M Brown, Int J Radiat Oncol Biol Phys , 9 (1983) 1513
4 C A. Barclay, J A Cerisola, H Lugones and O Ledesma, in W Siegenthaler and R Lathy (Editors), Current Chemotherapy, Vol 1, American Society for Microbiology, Washington, DC, 1978, p 158
5 J T Roberts, N M Bleehen, F Y F Lee, P Workman and M I Walton, Int J Radiat Oncol Biol Phys , 10 (1984) 1745
6 F Y F Lee and P Workman, Br J Cancer, 47 (1983) 659
7 J M Brown, Int J Radiat Oncol Biol Phys , 8 (1982) 675

8 I J Stratford and G E Adams, in A Breccia, B Cavalleri and G E Adams (Editors), Nitroimidazoles, Chemistry, Pharmacology and Clinical Application, NATO Advanced Study Institute Series, Series A Life Sciences, Vol 42, Plenum, New York, 1982, p 67
9 J Raaflub and W H Ziegler, Arzneim -Forsch , 29 (1979) 1611
10 P Workman, R A S White, M I Walton, L N Owen and P R Twentyman, Br J Cancer, 50 (1984) 291
11 I R Flockhart, P Large, D Troup, S L Malcolm and T R Marten, Xenobiotica, 8 (1978) 97
12 A J Varghese, S Gulyas and J K Mohindra, Cancer Res , 36 (1976) 3761
13 P Workman and P R Twentyman, Br J Cancer, 46 (1982) 249