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Note

Analysis of the basic 5-nitroimidazole nimorazole in blood by reversed-phase high-performance liquid chromatography, and its application to pharmacokinetic studies in individual mice

PAUL WORKMAN

MRC Clinical Oncology and Radiotherapeutics Unit, The Medical School, Hills Road, Cambridge CB2 2QH (Great Britain)

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Nitrimidazole derivatives are widely used clinically as antimicrobial agents [1] and to sensitise hypoxic tumour cells to radiation [2]. In the latter case, monitoring of plasma or saliva drug concentrations has been used to estimate the potential toxic and therapeutic effects [3, 4].

The in vivo concentrations of two of the more widely used drugs, misonidazole (Roche, Welwyn Garden City, Great Britain) and metronidazole (May & Baker, Dagenham, Great Britain), can be determined by reversed-phase high-performance liquid chromatography (HPLC) [5, 6]. As part of a series of studies in this laboratory on the pharmacokinetics of hypoxic cell radiosensitisers, it has been found that whereas the above HPLC methods are readily adapted for the assay of other essentially unionised nitroimidazoles, the method is often highly unsatisfactory for more strongly ionised derivatives. These latter include a class of compounds of particular interest, the basic morpholine derivatives, e.g. nimorazole, used clinically mainly as an antitrichomonal agent [7] (Fig. 1).

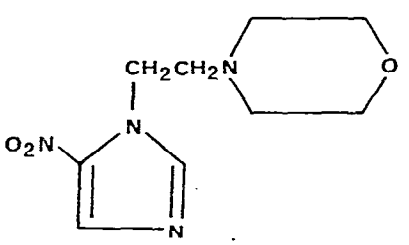


Fig. 1. Structural formula of nimorazole.

Reversed-phase HPLC, with aqueous methanol as the mobile phase and octadecylsilane as the stationary phase, gave poor chromatography with irreproducibility, broad asymmetrical peaks and poor resolution as the major problems. In the present paper a method is described for the estimation of nimorazole in whole blood by paired-ion reversed-phase HPLC [8].

In addition, the procedures were adapted to micro-scale to allow rapid analysis of 5-μl blood samples collected sequentially from individual mice, thus allowing detailed pharmacokinetic studies to be carried out with small quantities of drug. An application of the method is described, which demonstrates the dose-dependent pharmacokinetics of nimorazole in mice.

METHODS

Reagents

Nimorazole (4-[2-(5-nitroimidazol-1-yl)ethyl]-morpholine; nitrimidazine; Nagoxin) was supplied by Montedison Pharmaceuticals (Barnet, Great Britain), and 1-(2-nitroimidazol-1-yl)-3-chloropropan-2-ol (Ro 07-0269) by Roche.

Water was twice-distilled in glass. HPLC-grade methanol was obtained from Rathburn Chemicals (Walkerburn, Great Britain). Heptanesulfonic acid was obtained as a commercial preparation in aqueous solution containing acetic acid (PIC Reagent B-7) from Waters Assoc. (Milford, Mass., U.S.A.). The mobile phase was prepared by dissolving 15 ml of this reagent in 1 l of 30% methanol in water. This gave a heptanesulphonic acid concentration of 5 m*M* and a pH of 3.2. The mobile phase was passed through a 0.45-μm Millipore filter (for aqueous solvents) and de-aerated under vacuum before use.

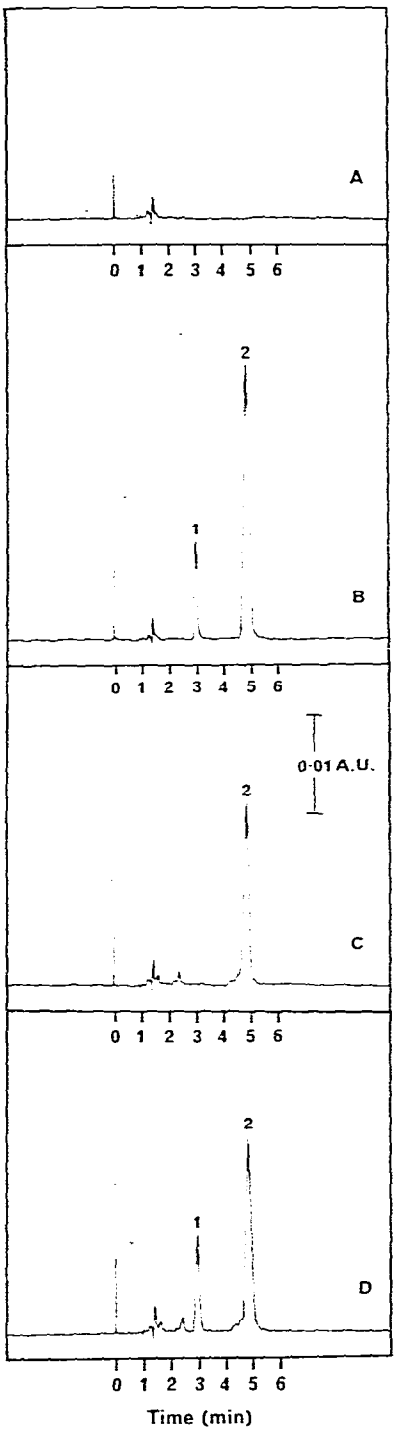
Sample preparation and chromatography

Blood samples (whole or diluted) were mixed thoroughly with 9 vol. methanol containing the standard Ro 07-0269 (11 μg/ml). After centrifugation (3000 *g*, 10 min), 10-μl samples of supernatant 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.). The mobile phase consisting of methanol—water (30:70) containing PIC Reagent B-7 was delivered at a constant flow-rate of 2 ml/min (pressure 20 MPa or 3000 p.s.i.). The absorbance of the column effluent was monitored at 313 nm using a Waters Model 440 absorbance detector coupled to a Servoscribe chart recorder (chart speed 10 mm/min).

Pharmacokinetics

Adult male BALB/c mice, weighing approx. 25—30 g were obtained from the breeding colony at N.I.M.R. (Mill Hill, London, Great Britain). They were allowed PRD nuts (Labsure Animal Diets, Poole, Great Britain) and tap water, both ad libitum.

For pharmacokinetic studies nimorazole was dissolved at appropriate concentrations in Hank's balanced salt solution and injected intraperitoneally in a volume of 40 ml/kg body weight. In the initial studies mice were bled at appropriate times by cardiac puncture under diethyl ether anaesthesia, and


A
0 1 2 3 4 5 6
2
1
B
0 1 2 3 4 5 6
0·01 A.U.
2
C
0 1 2 3 4 5 6
2
1
D
0 1 2 3 4 5 6
Time (min)

duplicate samples of heparinised whole blood (usually 0.1 ml) were then analysed. Subsequently, however, the method was scaled down to allow the analysis of sequential blood samples taken from the tail of individual mice. Duplicate 5-μl blood samples were collected in Microcap pipettes (Drummond, Broomall, Pa., U.S.A.) and mixed with 45 μl water. They were stored at −20° and analysed as described above, with the Ro 07-0269 standard added in the methanol.

Ten duplicate 5-μl samples add up to a total volume of 0.1 ml. This represents about 5% of the blood volume in the mouse.

RESULTS

Fig. 2A shows the chromatogram of a methanol extract of pooled, control undiluted whole blood from a group of BALB/c male mice; the standard Ro 07-0269 was omitted. Similar results were obtained for extracts of whole blood collected at various times after mice received Hank's balanced salt solution, the vehicle used for nimorazole injection.

The chromatogram in Fig. 2B is that of a methanol extract of control whole blood spiked with nimorazole (peak 2) and containing the Ro 07-0269 standard (peak 1). These had retention times of 4.9 min and 3.0 min respectively, and the corresponding capacity factors were 3.27 and 2.00 respectively. Coefficients of variation for the retention times, calculated from 64 samples over 2 days, were <1% for both nimorazole and the Ro 07-0269 standard.

Fig. 2C and D show chromatograms of methanol extracts of whole blood from a mouse which received nimorazole (1 g/kg), by intraperitoneal (i.p.) injection in Hank's balanced salt solution, 4.3 h previously; the standard was omitted from Fig. 2C but included in Fig. 2D. Both chromatograms contain a peak corresponding to nimorazole, and also contain several smaller peaks which may be nimorazole metabolites. Comparison of Fig. 2A—D shows that the control blood contains no interfering peaks, and that the internal standard is resolved from the parent drug and its putative metabolites.

To determine the efficiency of extraction of nimorazole from whole blood, samples of blood and of water, containing various known concentrations of nimorazole, were analysed as described for blood in the Methods section. Comparison of the data for blood and water samples showed that over the concentration range studied (5—1000 μg/ml) the extraction efficiency was 100%. The extraction efficiency of the Ro 07-0269 was also 100%. A calibration plot of normalised peak height ratio (peak height nimorazole:peak height standard) against blood nimorazole concentration was linear over the range 5—1000

Fig. 2. HPLC chromatograms of methanol extracts of undiluted whole blood from BALB/c male mice. Chromatographic conditions: column, μBondapak C_{18} (30 cm × 3.9 mm I.D.; particle size 10 μm); mobile phase, methanol-water (30:70) containing PIC Reagent B-7 (Waters Assoc.); flow-rate, 2 ml/min; column pressure, 20 MPa or 3000 p.s.i.; temperature, ambient; detection, absorbance at 313 nm; sample volume, 10 μl; chart-speed, 10 mm/min. (A): Control whole blood. Ro 07-0269 standard omitted. (B): Whole blood spiked with nimorazole (500 μg/ml; peak 2). Peak 1 corresponds to the Ro 07-0269 standard (11 μg/ml methanol). (C): Whole blood from a mouse injected with 1 g/kg nimorazole i.p., 4.3 h previously. Ro 07-0269 standard omitted. (D): As (C) but contains Ro 07-0269 standard.

μg/ml (r = 0.999, p <0.001) and the intercept on the ordinate was not significantly different from zero (p >0.1).

The lower limit of sensitivity for nimorazole, as defined by a signal-to-noise ratio >3, was approx. 5 ng. Thus for a 10-μl injection the lower limit of sensitivity for nimorazole in undiluted whole blood was about 5 μg/ml. For the microprocedure the lower limit was approx. 50 μg/ml. The sensitivity could be improved by concentrating the methanol extract, but this proved unnecessary for the present studies.

Coefficients of variation were calculated for the ten independent replicate assays of the same blood sample. In a test to compare the coefficients of variation for the whole blood assay at different nimorazole concentrations, values obtained were 3.4% at 500 μg/ml and 3.9% at 33 μg/ml. In a separate test to compare the coefficients of variation for the two procedures, values

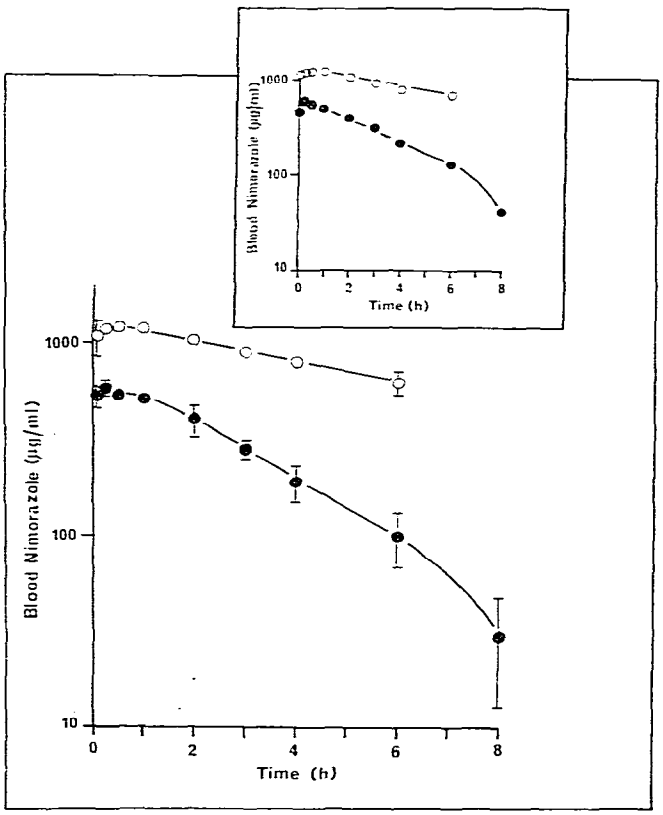


Fig. 3. Blood concentrations of nimorazole in BALB/c mice after i.p. injection of 1.31 g/kg (5 mmoles/kg, ○) or 0.655 g/kg (2.5 mmoles/kg, ●). Three mice were studied at each dose; error bars show 2 S.E. Inset: data for a single mouse at each dose.

obtained were 5.1% for the whole blood assay and 3.4% for the microprocedure (500 μg/ml nimorazole in whole blood).

Typical values for height equivalents to a theoretical plate were 0.11 mm for both nimorazole and Ro 07-0269.

The HPLC method described above was used to investigate the pharmacokinetics of nimorazole in BALB/c male mice. Fig. 3 shows the mean concentrations of nimorazole in whole blood at various times after i.p. injection of 1.31 g/kg (5 mmoles/kg) and 0.655 g/kg (2.5 mmoles/kg) (three mice per group). Absorption from the peritoneum into the circulation is rapid and the elimination comparatively slow. Moreover, the elimination half-life is dose-dependent, and the low-dose data seem to reveal a deviation from first-order elimination kinetics.

The scaling down of the method allowed the pharmacokinetics to be determined in individual mice (e.g. see inset, Fig. 3). Apparent half-lives were calculated for data from 1—6 h inclusive using the method of least squares linear regression analysis. The values obtained were 2.47 h, 1.76 h and 2.01 h for the lower dose, and 6.89 h, 5.18 h and 4.77 h for the higher dose.

DISCUSSION

The advantages of HPLC for the assay of nitroimidazoles have been described previously [5]. Conventional reversed-phase HPLC was found to be unsuitable for the analysis of ionised nitroimidazoles, and the present paper describes a paired-ion method for the rapid analysis of the basic 5-nitroimidazole nimorazole present in whole blood at pharmacological or toxicological concentrations. The particular advantages of this method over conventional reversed-phase HPLC with methanol—water mixtures as the mobile phase are (1) improved reproducibility, (2) improved performance and peak symmetry and (3) increased resolution. Chromatograms from mice receiving nimorazole contained several small peaks not present in control samples. These were not seen using conventional reversed-phase HPLC. Although the peaks have not been identified it is likely that at least some of these are nimorazole metabolites. Two urinary metabolites have been found in man [7].

As would be expected, the method is not suitable for strongly acidic nitroimidazoles ($pK_a < 3$), e.g., some carboxylic acid derivatives and glucuronide metabolites. However, good results have been obtained using paired-ion chromatography at neutral pH with tetrabutylammonium phosphate as the counter-ion [9].

There is at the moment considerable interest in the design, synthesis and testing of new nitroimidazole compounds for use as radiosensitisers in the treatment of cancer [2, 10]. Most in vivo studies are carried out on mice, and comparative pharmacokinetic investigations in this species should represent an important aspect of the drug development programme. The sensitivity of the present HPLC method has allowed the sample preparation to be adapted to the

analysis of 5-μl blood samples and even less. This has two important advantages. Firstly, it allows sequential blood samples to be taken from the same mouse, and negates the need for anaesthetics and for the animal to be sacrificed at sampling. This allows pharmacokinetic investigations in individual mice, if necessary on several different occasions. Some differences in nimorazole half-life between individual mice are shown in the present paper.

The second advantage of this technique is that considerably less drug is required to obtain the necessary pharmacokinetic data. This is particularly important in the screening of nitroimidazole radiosensitisers, which have to be given in comparatively large doses, often greater than 5 mmoles/kg. Drug development costs could therefore be reduced with this technique.

The present HPLC method has been used to show that the elimination of nimorazole from mouse blood does not obey first-order kinetics, and that longer half-lives are seen at higher doses. Similar results have been obtained for these 2-nitroimidazole misonidazole in mice [11]. This demonstration of dose-dependent elimination kinetics has important implications for the selection and subsequent use of nitroimidazole misonidazole radiosensitisers.

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