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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE NEW HYPOXIC CELL RADIOSENSITISER, Ro 03-8799, IN BIOLOGICAL SAMPLES

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SUMMARY

A high-performance liquid chromatographic method of analysis with UV detection has been developed to measure levels of a new radiosensitiser, Ro 03-8799 and its N-oxide metabolite, in biological fluids and tissues.

The accuracy and precision of the method have been determined in both plasma and urine, where the limits of quantitation are 100 and 500 ng/ml, respectively. Typical results are presented from a human volunteer study where samples were analysed by this method.

Important aspects of the method, involving both sample handling techniques and chromatographic conditions are discussed.

INTRODUCTION

Ro 03-8799, 2-nitro-α-(piperidino-methyl)-1-imidazole ethanol, is one of a series of 2-nitroimidazoles which are hypoxic cell radiosensitisers. These are compounds which enhance the effects of ionising radiation during radiotherapy of various tumours. During radiation the hypoxic cells in tumours are resistant to X-rays [1, 2] and only the oxic cells are killed. Nitroimidazoles form radical anions which render the hypoxic cells susceptible to the radiation [3, 4].

Because large doses are needed to achieve sufficiently high tumour concentrations, the effectiveness of radiosensitisers is limited by their toxicity. Misonidazole is one of the more effective radiosensitisers, and has been used in clinical trials for a number of years, but the doses that can be administered are limited by peripheral neuropathy [5, 6]. Ro 03-8799 is currently being evaluated in the clinic as a more potent and less toxic successor to misonidazole.

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A rapid and sensitive method to measure Ro 03-8799 (Fig. 1) in biological samples has therefore been developed for use in these trials. Since metabolic studies have shown (unpublished work) that the N-oxide (Ro 31-0313) is formed as a metabolite in six species studied, including man, we have developed a method which is capable of measuring both unchanged drug and N-oxide. Also, because the effectiveness of radiosensitisers depends on their penetration into tissues and therefore tumours, it was felt that the analytical method should be capable of quantifying these compounds in tissues and tumours as well as biological fluids.

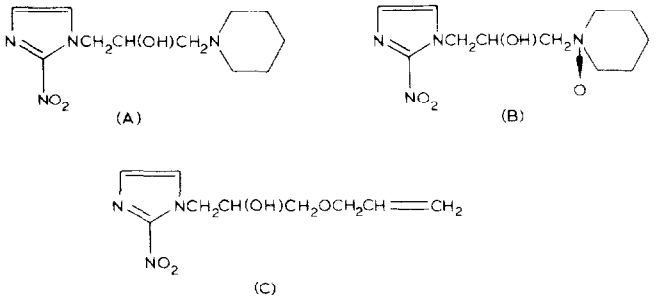

Fig. 1. Structures of Ro 03-8799 (A), Ro 31-0313 (B) and Ro 07-1902 (C).

MATERIALS AND METHODS

Chromatography

The samples are injected onto a Waters μBondapak ODS (10% coating) silica column (25 × 0.4 cm I.D., particle size 10 μm) by a Waters sample processor (WISP). The column is connected to an LDC Spectromonitor III, the wavelength being set at 326 nm. The UV absorbance is recorded by a Hewlett-Packard 3385 integrator and peak areas are used for quantitation. The eluent is 15% acetonitrile in glycine—hydrochloric acid buffer, pH 2.6, containing 3 m*M* heptanesulphonic acid. The flow-rate is kept constant at 1.3 ml/min using a Constametric Model III pump.

Biological samples

Plasma and erythrocytes. Blood samples are transferred into heparinised tubes, plasma and red cells separated by centrifugation and each stored frozen until analysed. Plasma (200 μl) and red cells (ca. 200 mg) are added to centrifuge tubes containing an appropriate amount of internal standard (Ro 07-1902, see Fig. 1) in aqueous solution, then acetonitrile (2 ml) is added to precipitate the proteins. After centrifuging, the supernatant is transferred to a clean tube and blown to dryness under a gentle stream of nitrogen. The residue is taken up in eluent (100 μl) and an aliquot injected (50 μl) onto the high-performance liquid chromatographic (HPLC) column.

Bile and urine. After collection, samples are stored frozen until analysed. Urine (500 μl) and bile (100 μl) are added to centrifuge tubes containing an appropriate amount of internal standard solution (10—50 μl), diluted with water to 600 μl (urine) or 200 μl (bile), centrifuged and injected (10—50 μl) onto the HPLC column.

Tumours and tissues. At the time of collection, all samples should be immersed in methanol, cooled in an acetone—dry ice mixture (ca. —75°C) and stored at this temperature until analysed. In dealing with small rodents, samples are collected by opening up the peritoneal cavity of anaesthetised animals (diethyl ether), freezing the whole animal in liquid nitrogen, then removing the required tissues/organs and storing them in cold methanol as above. For analysis the samples are thawed in ice, water is added to make the total volume equal to five times the weight of tissue, and then they are homogenised using a Silverson mixer-emulsifier. The homogenate (200 μl) is added to a centrifuge tube containing an appropriate amount of internal solution (10—50 μl) and the sample made up to 300 μl with water. It is spun at > 30,000 *g* for 60 min and an aliquot of supernatant (50 μl) is injected onto the HPLC column. Some tumour samples are particularly hard and resistant to normal homogenisation. In these cases we have used a Spex freezer mill, which crushes the tissue at the temperature of liquid nitrogen. Methanol is then added and the sample processed as described above.

Quantitation

For each analysis, a series of known amounts of Ro 03-8799 and Ro 31-0313 are added to a blank plasma, urine, etc., and worked up at the same time and in the same way as the samples. Quantitation of unknowns is by interpolation from the standard curve of the peak area ratio (compound/internal standard) vs. the concentration of the standards.

RESULTS

Choice of method

Both drug and N-oxide metabolite are 1-substituted 2-nitroimidazoles and have intrinsic UV absorbance at 326 nm. The extinction coefficients (Fig. 2) of Ro 03-8799 and Ro 31-0313 are sufficiently high to be able to detect 1 ng of pure compound applied to the column, using the system described.

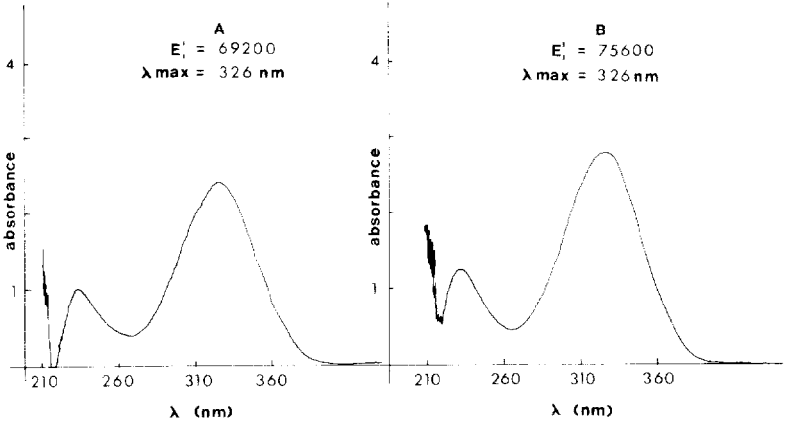

Fig. 2. UV absorbance of (A) Ro 03-8799, E_1' = 69200, λ_{max} = 326 nm and (B) Ro 31-0313, E_1' = 75600, λ_{max} = 326 nm, measured in eluent.

Ro 03-8799 is a lipophilic compound [7] (its octanol—water partition coefficient at pH 10.5 is 8.5) and is easily extracted from biological material at basic pH. This is not the case for Ro 31-0313 which, being an N-oxide, is a charged molecule. Therefore sample clean-up is restricted to removing the proteins. A reversed-phase HPLC column is used, because endogenous polar material remaining in the samples is eluted first, and therefore does not reduce column efficiency.

Both compounds have basic centres; the pK_a values of Ro 03-8799 and Ro 31-0313 are 8.7 and 4.7 [8], respectively. At eluent pH greater than 5 the peak shape of Ro 31-0313 is poor. Therefore we use a low pH with an ion-pair reagent (heptanesulphonic acid) to elute both basic compounds.

Plasma and red cells

Typical traces are shown in Fig. 3. To establish the accuracy and precision of the method, standard curves of parent drug and N-oxide covering the range 0.05—50 μg/ml were made up on four different occasions in blank human plasma. Results of these analyses showed that over the concentration range 0.1—50 μg/ml the precision of the method (mean ± S.D. of the calculated results) is < ± 5% and that the accuracy (comparison of the theoretical with the calculated concentration ± S.D.) is 100.2 ± 3%. Below 0.1 μg/ml these figures fall below acceptable limits. However, for this compound and its metabolite, therapeutic doses give concentrations well above this level.

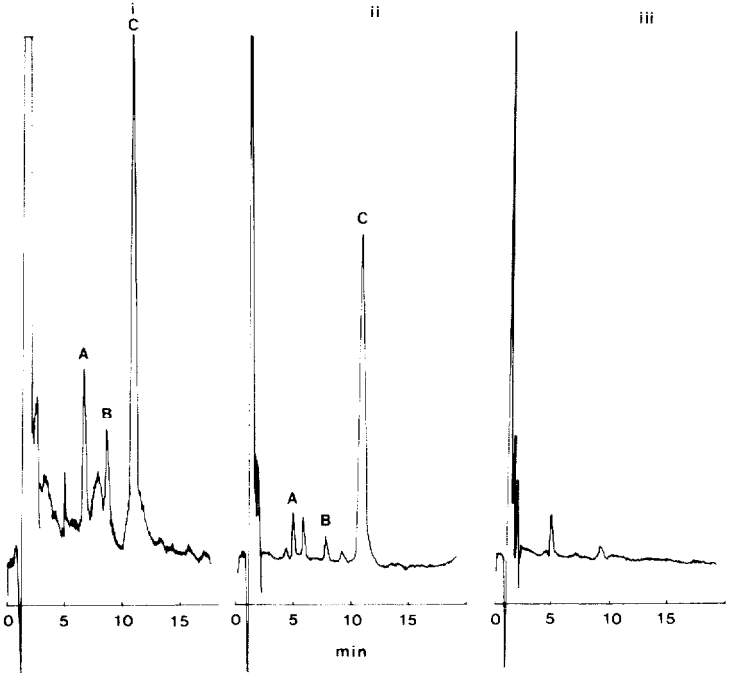

Fig. 3. Typical chromatograms from (i) volunteer patient 8-h plasma containing ca. 100 ng/ml Ro 03-8799 (A) and Ro 31-0313 (B) and internal standard (C), (ii) human plasma standard of 100 ng/ml A and B, with internal standard, C, and (iii) blank human plasma.

Using this method we have successfully analysed plasma samples from several species including man. A human volunteer study, in conjunction with Mount Vernon Hospital [9] was carried out, where volunteer patients were given intravenous infusions of [2-^{14}C]Ro 03-8799. Plasma, red cells, urine and tumour biopsies were all analysed for Ro 03-8799 and Ro 31-0313. Fig. 4 shows a typical concentration versus time plot of plasma and red cells. Tumour biopsies taken at the same time as three of the plasma samples all showed high levels of Ro 03-8799 (Table I). Although the biopsies were taken from the same tumour site, they were not identical samples [9] which could account for the variable tumour to plasma ratio.

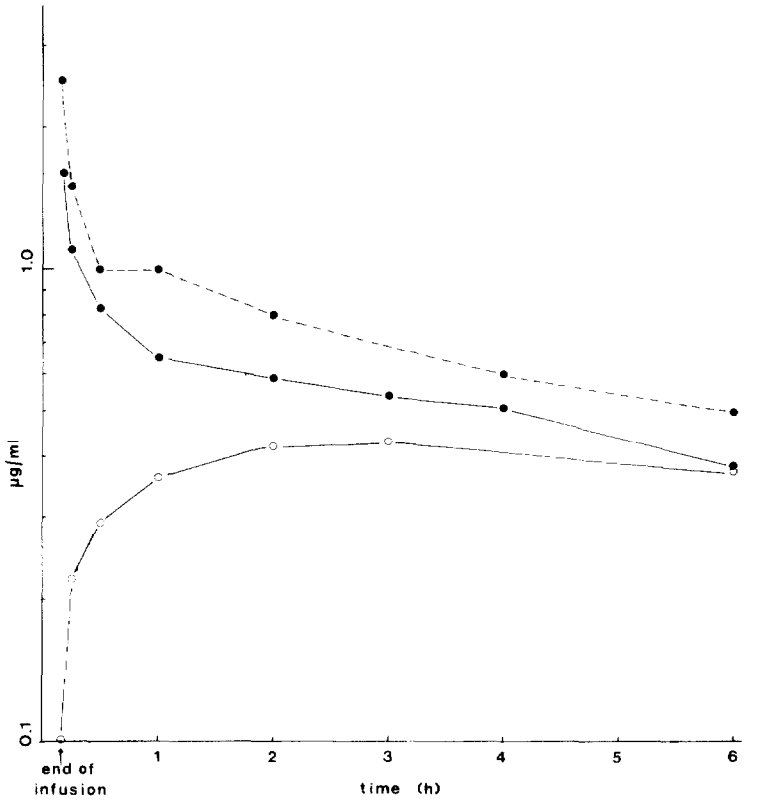

Fig. 4. Levels of Ro 03-8799 (•) and Ro 31-0313 (○) in plasma (——) and red cells (— — —) in a patient after an infusion of 100 mg of [2-^{14}C]Ro 03-8799.

Urine

Analysis of urine samples from animals fed on standard laboratory diets is straightforward. However, we have found considerable variation in human urine samples. There can be large and variable interfering peaks under both compounds depending on the source of the samples. This makes quantitation at low levels inaccurate. Standard samples of parent drug and N-oxide covering the range 0.4—50 µg/ml were made up on five different days. The precision and accuracy of the method were calculated as described for plasma. In the range 1—50 µg/ml the precision was $< \pm 10\%$ for both compounds, and the accuracy

TABLE I

COMPARISON OF LEVELS (μg/g) OF TOTAL DRUG-RELATED MATERIAL (^{14}C) AND Ro 03-8799 IN TUMOUR AND PLASMA FROM A PATIENT GIVEN A 100-mg INFUSION OF [2-^{14}C]Ro 03-8799

Time (h)	Tumour		Plasma	
	^{14}C	Ro 03-8799	^{14}C	Ro 03-8799
0.5	3.9	1.6	1.0	0.83
1.0	1.8	1.0	1.0	0.65
2.0	3.8	2.0	1.0	0.59

100 ± 2% for parent drug and 98 ± 4% for N-oxide. For all practical purposes the quantitation limit is 0.5 μg/ml where the precision is ± 20%. For the quantitative analysis of human urine samples therefore, it is important to collect blank samples corresponding to those being analysed.

Tissues

A tissue distribution study was carried out in rats dosed with [2-^{14}C] Ro 03-8799. Initially samples were removed from the animals and stored frozen until analysed. Although the levels of drug-related material, measured by radioactivity, were high (3—10 times plasma levels at 15—60 min) we were unable to account for any of this material as unchanged drug or N-oxide metabolite. By altering the conditions under which the tissues are removed from the animals, to that given in the Experimental section, we increased the amount recovered as Ro 03-8799 at 30 min after dosing to 60% in all tissues apart from liver. 35% of the radioactivity measured in the liver can be shown to be unchanged drug (30%) and N-oxide metabolite (5%).

DISCUSSION

The analytical method described has proved very successful in analysing Ro 03-8799 and its N-oxide. The procedure has been applied to various biological samples and there is no interference from other metabolites. To obtain reproducible results however, certain aspects of the method are critical.

The pH of the eluent is important when analysing plasma samples because at a pH of 7 or greater no N-oxide is detected. This is because it is not present as such in the general circulation, but is immediately formed under acidic conditions (pH < 7). Measurement of the authentic metabolite as the N-oxide is considered to be justified since this allows all the drug-related material in human plasma to be determined. Also the N-oxide is present in urine which is slightly acidic.

The precautions described for handling tissue samples are necessary to prevent ex vivo metabolism. Ro 03-8799 was designed as an electron-affinic compound and is therefore an ideal substrate for nitro-reductase enzyme systems [10, 11]. In the anoxic conditions prevailing during tissue removal, the nitroimidazole can be rapidly reduced to a complex mixture having no characteristic UV absorption.

Using the procedures described, samples from the preliminary study in humans have been successfully analysed. The simple clean-up coupled with the automated HPLC analysis makes it possible to process large numbers of samples easily and quickly. The method should therefore be suitable for use by various laboratories where clinical trials on this new radiosensitiser are being carried out.

ACKNOWLEDGEMENTS

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