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Note

Antitumour imidazotetrazines

VII. Quantitative analysis of mitozolomide in biological fluids by high-performance liquid chromatography

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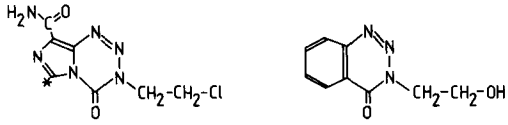
The novel agent mitozolomide, 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one, also designated CCRG 81010, M&B 39565 and NSC 35451, see I in Fig. 1, was synthesised in our laboratories [1] as a part of a programme concerned with investigating the chemical and biological properties of molecules containing NNN linkages [2—4]. The compound was found to be active against a wide range of model tumour systems [1, 5] and mechanistic studies [6—8] have indicated that mitozolomide may be a prodrug of the chemically unstable monochloroethyltriazene, 5-[3-(2-chloroethyl)-triazen-1-yl]imidazole-4-carboxamide (MCTIC). Preliminary animal pharmacokinetic data [9] indicated that mitozolomide was well absorbed orally (in mice), and had an elimination half-life of just over 1 h. In order to investigate the drug's in vivo and in vitro kinetics a rapid, sensitive and selective high-performance liquid chromatographic (HPLC) method was developed.



I II

Fig. 1. Structural formulae of mitozolomide (I) and the internal standard (II), 3-(2-hydroxyethyl)-1,2,3-benzotriazin-4(3H)-one. The star denotes the position of ^{14}C in mitozolomide.

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EXPERIMENTAL

Materials

Mitozolomide was kindly supplied by Dr. E. Lunt of May & Baker (Dagenham, U.K.) as was the [^{14}C]mitozolomide (see Fig. 1 for position of label). The internal standard, 3-(2-hydroxyethyl)-1,2,3-benzotriazin-4(3H)-one, was synthesised by G.U. Baig, All chemicals and solvents were of either analytical or chromatographic quality and were used without further purification.

Extraction

Plasma samples were stored at −20° C until required. A 20-μl aliquot of the internal standard solution was added to a 10-ml test tube followed by 0.05 ml of 1 *M* hydrochloric acid and 1 ml of plasma. Ethyl acetate (2.5 ml) was added, the contents mixed by vortexing and the layers separated by centrifugation (10 min at 1500 *g*). A 2-ml aliquot of the organic layer was removed and evaporated to dryness using a stream of dry nitrogen. The residue was dissolved in 0.15 ml of methanol and 0.15 ml of 5% acetic acid in water was immediately added. This solution was transferred to a low volume insert and stored at 4° C until analysed (within seven days).

Calibration curves were constructed over the range of interest by the addition of chloroform solutions of mitozolomide to test tubes and evaporating to dryness using a stream of dry nitrogen. The procedure was then as described above.

Chromatography

A 100 × 5 mm Waters RCM cartridge (10 μm particle size, C_{18}) was used with a C_{18} pre-column. The isocratic mobile phase consisted of methanol—5% acetic acid in water (3:7) and was pumped at a constant flow-rate of 1.5 ml/min. A Waters system was used (Waters Assoc., Northwich, U.K.) which comprised of a WISP Model 720 system controller and M730 data module. The injection volume was 20 μl and detection was at 325 nm using a Waters Lambda-max 480 LC spectrophotometer.

Extraction efficiencies

Aqueous solutions of [^{14}C]mitozolomide were added to control plasma at 1, 5, 10 and 20 mg/l. Following extraction with ethyl acetate both plasma and ethyl acetate samples were counted on a Beckman LS230 liquid scintillation counter. Values (dpm) were calculated using an external standard channels ratio method. The scintillant used was Fisofluor (Fisons, Loughborough, U.K.).

Quantification of mitozolomide

The concentration of mitozolomide was determined from the peak area ratio of mitozolomide to internal standard. Calibration curves were constructed over the range of interest using at least six evenly distributed points and the data analysed by least-squares regression.

RESULTS AND DISCUSSION

Mitozolomide was quantified by HPLC following ethyl acetate extraction of acidified plasma. The extraction efficiencies were determined using ^{14}C-labelled mitozolomide which had been added, at various concentrations, to 1 ml of control plasma. The results indicated that the extraction into 2.5 ml of ethyl acetate was constant, over the range studied, at 76% with 95% of the total label being recovered. The effect of the addition of 0.05 ml of 1 *M* hydrochloric acid on the extraction efficiency was determined and was shown to be negligible. The coefficient of variation for the replicate extractions (n = 6) of plasma samples was 1.8% and for replicate injections of the same sample was 0.5% (n = 6). The detection limit of the assay was 10 ng/ml with a signal-to-noise ratio of greater than 3.0.

The capacity factors for mitozolomide and the internal standard were 1.5 and 2.6, respectively, which gave a total analysis time of 5 min. Typical chromatograms are given in Fig. 2. The chemical stability of mitozolomide in various buffers and physiological fluids was examined and the results are summarised in Table I. The aqueous protein solution was bovine serum albumin at the same concentration as total protein in normal human plasma, 70 mg/ml [10].

Potential difficulties associated with the quantitative analysis of mitozolomide centred on the chemical instability of the compound. However,

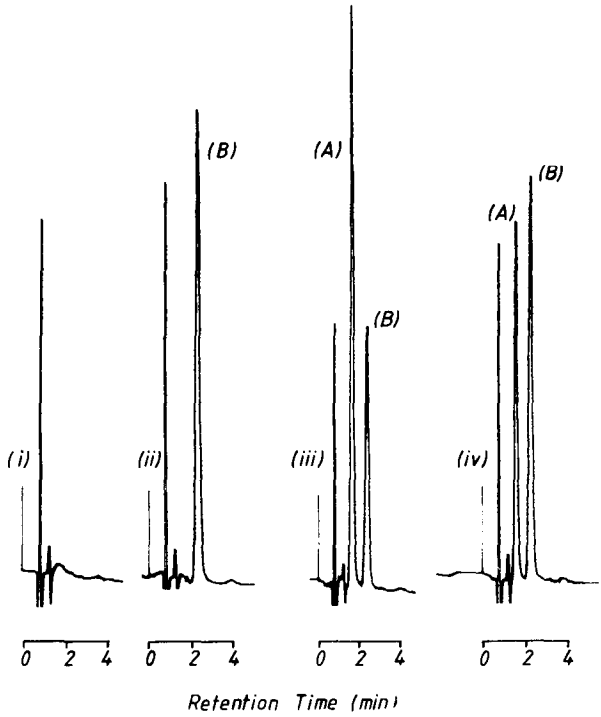

Fig. 2. Chromatograms of mouse plasma extracts. (i) Blank plasma; (ii) blank plasma spiked with internal standard; (iii) blank plasma spiked with 1 μg mitozolomide and internal standard; (iv) plasma from mouse dosed with 20 mg/kg of mitozolomide and extracted with internal standard. Peaks: A = mitozolomide; B = internal standard.

TABLE I

CHEMICAL STABILITY OF MITOZOLOMIDE

Solution	pH	Temperature (°C)	Half-life (h)
Tris buffer (0.1 m*M*)	9.0	22	0.15
Phosphate buffer (0.07 *M*)	7.4	37	0.92
Acetate buffer (0.1 *M*)	4.0	37	240
Bovine serum albumin (70 mg/ml)	7.4	37	0.87
Human plasma	7.4	37	0.48
Human urine	6.2	37	32.8

due to the high stability at low pH (see Table I), it was apparent that, as long as the blood or urine was cooled immediately following collection and that extraction conditions were kept acidic, there were no undue problems in this area. The strong chromophore at 325 nm [1] facilitated interference-free traces to be obtained from control plasma (Fig. 1) and has enabled pharmacokinetic analyses to be performed at doses which correspond to 1/100 of the optimum therapeutic dose for mice innoculated with model tumours [1]. The HPLC assay described above has been used to determine pharmacokinetic parameters of the drug in over 40 patients involved in a phase I trial of mitozolomide. There was no evidence from the pre-dose plasma samples, that other drugs which would commonly be coadministered with this agent (e.g. antiemetics and analgesics) interfere with the assay.

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