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Determination of altretamine in human plasma with high-performance liquid chromatography

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Abstract

A fast, simple, and sensitive isocratic HPLC method has been developed and validated for the determination of the anticancer drug altretamine in human plasma. Spiked serum samples and clinical plasma samples are extracted with acetonitrile at 4°C and the precipitate removed by filtration. The plasma sample volume required (ca. 0.2 ml) is small and the total analysis time is less than 15 min per sample (including batch-wise pre-treatment). Recovery of altretamine is 99 to 106% for pooled human serum spiked with altretamine in the range 200 ng/ml to 10 mg/ml. In this concentration range, the R.S.D. varies from 1 to 8%. The limit of quantitation is ca. 150 ng/ml for an R.S.D. of 10%. The intra-day R.S.D. for human samples spiked at 5 mg/ml varied between 1.7 and 4%; the inter-day R.S.D. at this concentration was ca. 3%. A preliminary study with one patient receiving 260 mg/m^2 by mouth indicated that the peak altretamine concentration was significantly lower after a standard breakfast than in the fasting state.

1. Introduction

Altretamine is derived from the heterocyclic compound melamine, and is considered to act by alkylation of DNA following microsomal activation. It differs from the classical alkylating agents in that it is not completely cross-resistant with them [1]. Animal studies have shown that altretamine is quickly distributed amongst tissues and demethylated in the liver [2]. Moreover, cell culture studies have shown that metabolism of altretamine is necessary for antitumour activity to occur [3], although the ultimate therapeutic agent remains elusive. Its use in human cancer is largely in the context of ovarian cancer [4] and studies by the current authors and others are currently under way in the UK, exploring its activity after relapse following first-line treatment.

One of the advantages of altretamine is its activity when administered orally, thus reducing the time the patient spends in hospital and thereby having the potential to enhance quality of life. However, a major problem with orally administered drugs in general is that absorption may be variable and that food may interfere with this absorption. This is a particular problem with anticancer drugs which have a low therapeutic

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index. Thus efficacy may be significantly compromised if the effect of a dose selected as being tolerable from the point of view of toxicity is diminished by poor absorption in the presence of food. Alternatively, drug absorption may be enhanced and delayed by the presence of food [5].

Earlier, D'Incalci et al. [6] investigated the pharmacokinetics of orally administered altretamine. They found no correlation between the dose (120–300 mg/m^2) and peak plasma concentration C_{max}, which varied between 0.2 and 20.8 μg/ml for 11 cancer patients. Thus the level of altretamine was found to be variable, and sometimes found to be very low. In another study by D'Incalci et al. [7], patients with ascites, which occurs in 50% of patients with advanced ovarian cancer, showed less variation in plasma level of altretamine than those without ascites. The variable and frequently low levels of altretamine observed could be mainly attributable to differences in liver function, rather than to poor absorption from the gut [8]. Patients with metastatic cancer may have impaired liver function, so that variable rates of altretamine metabolism could be anticipated.

In current studies on the effect of food on the absorption of altretamine, it was necessary to develop a fast, simple, and sensitive method to permit drug levels to be monitored as a function of time. A number of HPLC methods reported earlier [9,10] have been applied in animal studies only. The present method has been developed and validated specifically for the assay of altretamine in human plasma.

2. Experimental

2.1. Reagents and materials

Methanol and acetonitrile were HPLC grade and purchased from Fisons Scientific Equipment, Loughborough, UK. Phosphate buffer was prepared from analytical reagent-grade sodium dihydrogen phosphate dodecahydrate (3.14 g) and disodium hydrogenphosphate dihydrate (0.108 g) (Fisons Scientific Equipment) dissolved in 500 ml double-distilled and deionised water and made up to 1 l. This produced a solution of pH 8.2 and a total phosphate concentration of 10 m*M*. The mobile phase consisted of MeOH–phosphate buffer (70:30, v/v).

Altretamine was kindly provided by US Biosciences, Watford, UK. Pentamethylmelamine and 2,2,4,6-tetramethylmelamine were kindly donated by Dr. M. Ames (Mayo Clinic, Rochester, MN, USA). Altretamine was formulated in 50-mg gelatin capsules. Pooled human male AB (whole blood) serum (catalogue No. H1513) was purchased from Sigma, Poole, UK. The clinical plasma samples were taken from ovarian cancer patients at Airedale General Hospital (West Yorkshire, UK) with ethical committee approval. Hepflush was used as received from Leo Labs., Aylesbury, UK.

2.2. Sample collection protocol

Ovarian cancer patients taking part in this study had to fulfill certain criteria. Firstly, they had to have been previously treated with cisplatin and obtained a complete clinical response of at least six months duration. If a relapse occurred, they were treated with altretamine at a dose of 260 mg/m^2. Patients who agreed to take part in this study underwent the following procedure.

Patients attended Airedale General Hospital on two separate occasions having fasted and having not taken any medication from the previous midnight. On one occasion they were given a standard meal (breakfast) about 2 h before drug administration. Blood (1–2 ml) was taken via an in-dwelling cannula into lithium–heparin containers (Vacutainer, 10 ml; Becton Dickinson, Cowley, UK) immediately prior to drug administration. Blood was then taken at intervals of 15, 30, 45, 60, 75, 90, 120, 180, 240, 300 and 360 min after administering altretamine orally with water (ca. 100 ml). Samples were separated immediately and the plasma frozen at −20°C. Plasma was transported to the laboratory at the University of Bradford for analysis.

2.3. HPLC system

A modular HPLC system was used comprising of a Gilson 305 10SC pump (Villiers-le-Bel, France) which delivered mobile phase at 1 ml/min through an ODS1 column (250 × 4.6 mm) packed with C_{18} Spherisorb (5 mm) supplied by Hichrom (Reading, UK). A "direct-connect" guard column (Alltech, Carnforth, UK) with C_{18} packing (1 cm) was fitted to the inlet of the column. The Gilson 116 variable-wavelength UV detector was used at 230 nm for this work. A Rheodyne injector provided with a 20-μl loop was used; this was flushed with ca. five times the specified volume of each sample or standard. A Hewlett-Packard integrator (HP3394A; Stockport, UK) was used to record the chromatograms and integrate the peak areas.

2.4. Sample preparation

Spiking procedure

Stock solutions were prepared by dissolving altretamine in pure MeCN and diluting with water to 25% MeCN. No precipitation was discernable up to 500 mg/ml after dilution. A stock solution of 500 μg/ml in 25% MeCN was diluted to 250, 125, 62.5, 32.25 and 10 μg/ml for spiking human serum and water blanks. For validation purposes, human serum and water were spiked in the range 200 ng/ml–10 μg/ml by adding 0.1 ml of the appropriate solution to 4.9 ml of serum or water, respectively.

Extraction of spiked samples

Human serum (0.2 ml) and MeCN (0.6 ml) were cooled to ca. 4°C. The MeCN was slowly added to the serum in a Nalgene sample tube (2 ml) with a conical bottom. The white precipitate was vortexed for 10 s and filtered through a Sartorius RC4 syringe filter (Göttingen, Germany) into a sample tube and stored at 0°C prior to analysis. This procedure took ca. 40–50 min for a batch of 18 samples. Triplicate samples of spiked human serum at each concentration level were prepared for validation over the range 200 ng/ml–10 μg/ml ($n = 6$). One injection was made for each spiked sample. Inter-day reproducibility was tested by spiking Sigma human serum at 5000 ng/ml, and then extracting and analysing five samples (duplicate injections) on each of five days. Samples were stored at −20°C prior to analysis.

Blank 75% MeCN was occasionally injected to flush out the injector, which was washed with water and MeCN at the end of each analysis period. The column was washed with 75% MeOH at the end of each week before flushing with pure MeOH.

Extraction of clinical samples

Plasma samples (1–2 ml) obtained as described from patients after fasting and after the standard breakfast, were stored in a freezer at −20°C. Each sample was thawed out and extracted as described above for the spiked samples. Altretamine concentrations were calculated by reference to bracketting standards. It was established that the calibration curve passed through or close to the origin on the *y*-axis; the 95% confidence interval for the intercept, derived from the regression embraced the origin.

3. Results and discussion

3.1. Spiked standards

Regression analysis of the data for serum and water, each spiked with altretamine, showed an excellent correlation between peak area, I, and concentration, C_{altret}, at a detector sensitivity of 0.3 AUFS; the intercept (k) and slope (m) values were similar: $I = 104C_{\text{altret}} - 7820$ for human serum, and $I = 100C_{\text{altret}} - 7280$ for the aqueous standards. The correlation coefficients were 0.994 ($n = 6$) and 0.996 ($n = 6$), respectively. The calibration graph was shown to be linear in each case from 200 ng/ml to 10 μg/ml. The retention time of altretamine in this system ranged from 8.5–9.1 min, depending on ambient temperature. Over this range, the k' value was 3.9 ± 0.2.

The overall recovery approximated to 100% (ratio of the gradients for the calibration graphs for the spiked serum and aqueous sample), within the range 99–106%; R.S.D. varied from

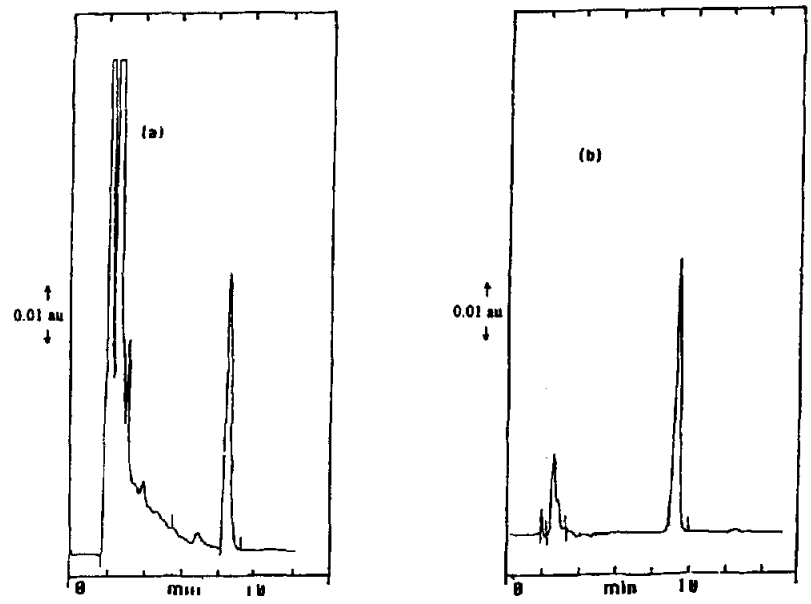

Fig. 1. Chromatograms of (a) Sigma human serum and (b) water, each spiked with altretamine at 5 μg/ml.

1–8% over this concentration range. In this respect, one outlier was observed at 625 ng/ml (recovery 80%; R.S.D. = 6.6%; $n = 3$) which could not be accounted for. The limit of quantitation (R.S.D. = 10%) was estimated at 150 ng/ml.

Fig. 1 shows typical chromatograms of extracted human serum and water spiked at 5000 ng/ml. The overall analysis time is ca. 12–15 min (including batch-wise sample preparation) and the altretamine peak is clearly separated from any endogenous plasma peaks. The chromatogram of the aqueous standard shown in Fig. 1b shows signs of disturbance at the solvent peak position due under the high sensitivity employed. The chromatogram of a pooled human serum blank shown in Fig. 2 confirms the absence of any potential interference under the altretamine peak. Measurement stability was checked at the beginning of each day by replicate injections and regularly monitored with aqueous standards. The intra-day R.S.D. for plasma spiked at 5 μg/ml ranged from 1.7 to 4.0% over 5 days.

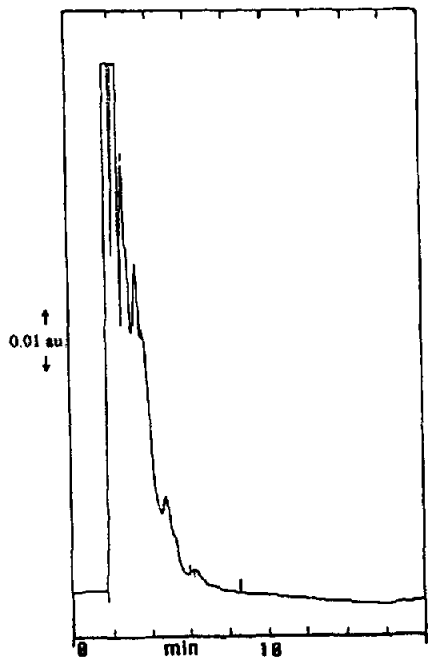

Fig. 2. Chromatogram of extract from blank Sigma human serum.

The results of an inter-day study show that the method has a high degree of reproducibility; the R.S.D. for a similar plasma standard was 2.8% ($n = 5$); the mean recovery at 5 μg/ml observed over five days corresponded to 96% ($n = 51$).

3.2. Clinical samples

Fig. 3 shows the chromatogram of a clinical sample from a patient J.L. taken 30 min after dosing, compared with that of a standard aqueous sample of altretamine (peak 1), together with the principal metabolites pentamethylmelamine (peak 2) and 2,2,4,6-tetramethylmelamine (peak 3). The altretamine peak is clearly separated from those of the metabolites and any endogenous components in the plasma. The retention times for the two metabolites (peaks 2 and 3) were ca. 5.2 and 6.8 min, respectively. A blank chromatogram of the pre-dose plasma sample of J.L. is shown in Fig. 4. In addition to altretamine, small amounts of pentamethylmelamine and tetramethylmelamine are apparent in the clinical sample.

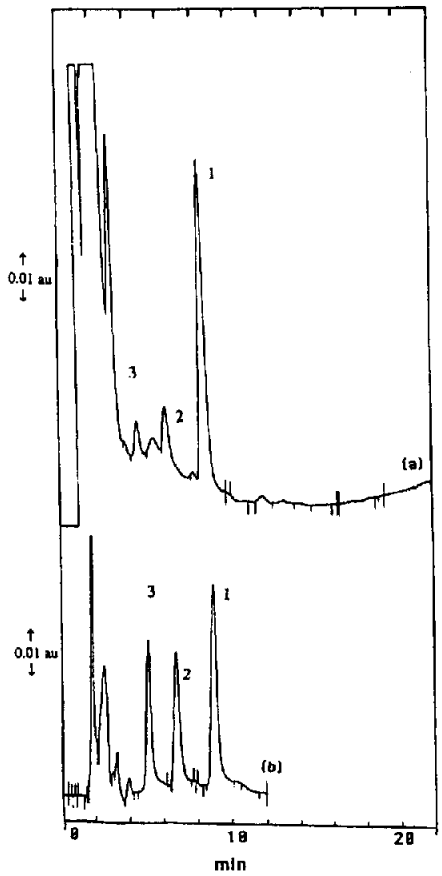

Fig. 3. Chromatograms of a clinical sample: (a) from patient J.L. and (b) a standard aqueous solution. Peaks: 1 = altretamine; 2 = pentamethylmelamine; 3 = 2,2,4,6-tetramethylmelamine.

As an example of the utility of this method, the altretamine concentration vs. time data for patient J.L. are presented in Fig. 5. The maximum plasma concentration (C_{max}) was 6270 ng/ml for the fasting period, and 3160 ng/ml after food, respectively. The higher peak plasma concentration observed under fasting conditions gives an early indication that there could be clinical problems in defining the appropriate dose for patients under non-fasting conditions.

This rapid method has proved to be a robust and sensitive procedure for clinical use in the treatment of ovarian cancer for research purposes. The method is sufficiently reliable for

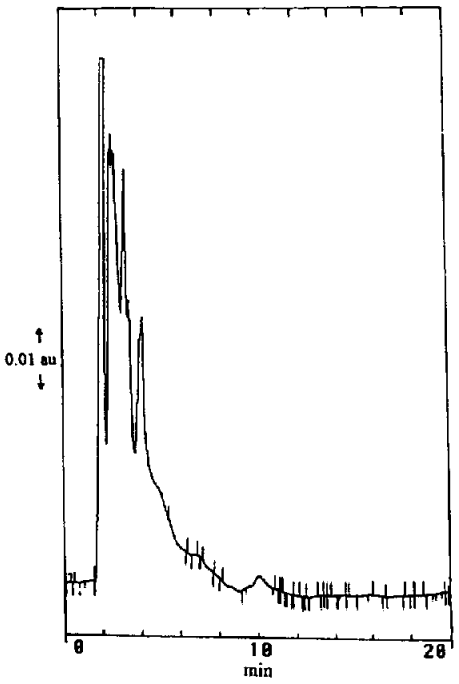

Fig. 4. Chromatogram of an extract from a pre-dose blank sample from patient J.L.

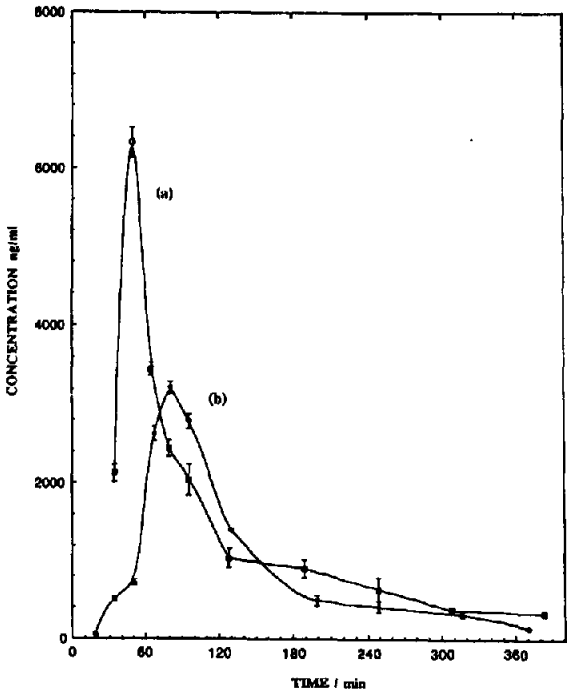

Fig. 5. Plasma altretamine concentrations of patient J.L., following oral administration of single dose of 260 mg/m^2: (a) after fasting (□) and (b) after food (◆).

studies on the pharmacokinetics in individual patients receiving altretamine under fasting and non-fasting conditions to be conducted with confidence.

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