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Short Communication

Bioanalysis of the investigational anti-tumour drug 5,10-dideaza-5,6,7,8-tetrahydrofolic acid by high-performance liquid chromatography with ultraviolet detection

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method with ultraviolet detection at 278 nm is presented for the determination of 5,10-dideaza-5,6,7,8-tetrahydrofolic acid in plasma. Sample pretreatment was achieved by using cation-exchange solid-phase extraction columns with methotrexate as internal standard. Chromatographic separation was based on ion-pair HPLC with 1-octanesulphonic acid as the ion-pairing compound. The detection limit was 10 ng/ml using an 500-μl sample volume. The assay was linear from the detection limit up to 5000 ng/ml with good reproducibility. The applicability of the assay was demonstrated in a study in the rat.

INTRODUCTION

(6*R*)-5,10-Dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF, lometrexol) (Fig. 1) is a novel antifolate antimetabolite, which displays a broad spectrum of activity against transplantable murine solid tumours and human tumour xenografts [1]. In contrast to classical antifolates such as methotrexate (MTX), the compound exerts cytotoxicity by inhibition of the key enzyme glycin-

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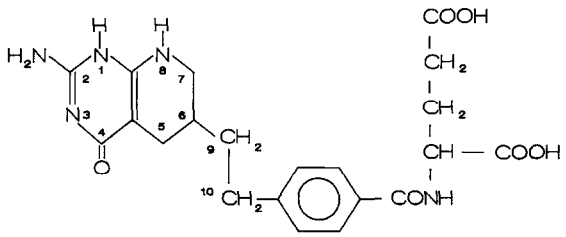

Fig. 1. Structure of DDATHF.

amide ribonucleotide formyltransferase, causing depletion of purine nucleotide pools and, consequently, impediment of *de novo* purine synthesis [2]. The toxicity of the compound is currently being investigated in phase I clinical trials. Subsequent phase II trials will give information about the therapeutic efficacy of the drug. The pharmacokinetic evaluation of a new entity constitutes an important part of a phase I study, and therefore the availability of a validated bioanalytical method is a prerequisite.

Up to now only a particle concentration fluorescence immunoassay (PCFIA) for the quantification of DDATHF in biological samples has been described [3]. Immunoassays, however, may lack specificity owing to cross-reactivity with structurally related compounds (*e.g.*, metabolites). Chromatographic techniques, in particular high-performance liquid chromatography (HPLC), are therefore generally preferred for pharmacokinetic monitoring during phase I trials, as these procedures may provide data on the metabolism of the compound. This paper is the first report of an HPLC bioanalysis of DDATHF.

EXPERIMENTAL

Materials and reagents

DDATHF was a generous gift from Dr. G. B. Grindey (Eli Lilly, Indianapolis, IN, USA). MTX was obtained from Sigma (St. Louis, MO, USA). All other reagents were purchased from E. Merck (Darmstadt, Germany) and were of analytical-reagent grade, except for acetonitrile, which was of HPLC grade. Water, purified with a Milli-Q system (Millipore, Bedford, MA, USA), was used throughout. Solid-phase extractions were performed on SPE–aromatic sulphonic acid ($C_6H_5SO_3H$) columns (J. T. Baker, Phillipsburg, NJ, USA). Drug-free human plasma was obtained from healthy volunteers.

Liquid chromatography

Chromatographic analyses were performed using an HPLC system consisting of a Spectroflow SF400 pump, a Spectroflow 757 UV–VIS detector operating at 278 nm with a response time of 2 s (Kratos, Ramsey, NJ, USA) and a Model 360 autosampler (Kontron, Basle, Switzerland). Samples were chromatographed on a glass column (100 × 3 mm I.D.) packed with 5-μm Nucleosil C_{18} material, preceded by a guard column (10 × 3 mm I.D.) packed with pellicular reversed-phase material (Chrompack, Middelburg, Netherlands). The mobile phase consisted of 803 ml of 50 m*M* KH_2PO_4 (adjusted to pH 3.5 with hydrochloric acid) and 40 ml of PIC B-8 reagent (Waters, Milford, MA, USA). This solution was filtered through 0.45-μm HAWP filters (Waters). Next, 157 ml of acetonitrile was added and the mixture was degassed by vacuum for 1 min. The mobile phase was kept well sealed to prevent evaporation. Elution was performed at 0.4 ml/min at ambient temperature. Integration was done using a WINner-4 data station (Spectra-Physics, San Jose, CA, USA).

Preparation of standard samples

A stock solution of 15 μg/ml DDATHF in water was stored at −20°C. Fresh standard solutions of DDATHF in water were prepared from this stock solution just prior to use. For the construction of a calibration graph in the range 20–250 ng/ml, a volume of 50 μl of DDATHF standard solution (concentration range 200–2500 ng/ml) was added to 500 μl of blank plasma, whereas for the calibration graph in the range 250–50000 ng/ml, a volume of 50 μl of DDATHF standard solution (concentration range 500–5000 ng/ml) was added to 50 μl of blank plasma.

Sample preparation

A 50-μl volume of internal standard (I.S.) (1.25

μg/ml MTX in water) and 500 μl of 5% (v/v) acetic acid in water were added to a volume of 500 or 50 μl of plasma (depending on the concentration of DDATHF). A solid-phase extraction column was preconditioned with 2 ml of methanol and 2 ml of 1% (v/v) acetic acid in water. The sample was applied to the extraction column. The column was rinsed with 2 ml of 1% (v/v) acetic acid and 1 ml of water and then dried by suction. Next, the compounds were eluted with 1000 μl of 500 m*M* phosphate buffer (pH 7.0). The eluate was adjusted to pH 3.5 with 5 *M* hydrochloric acid and an aliquot of 100 μl was subjected to chromatography.

Calibration

Ratios of peak areas of DDATHF and the internal standard were used for quantitative calculations. The calibration graphs were calculated by weighted linear regression analysis. Weighting was achieved by performing unweighted linear regression analysis after log–log transformation of the abscissa (concentration) and ordinate (ratio of DDATHF and internal standard) [4].

To minimize the amount of sample needed, two calibration ranges, utilizing 500 μl for low concentrations and 50 μl for higher concentrations, were used.

Validation

The accuracy of the method at each standard concentration was judged from the percentage relative concentration residuals (%*RCR*), which is defined as $\%RCR = 100(RC-NC)/NC$, where *RC* and *NC* represent the interpolated and nominal concentrations, respectively. The within-run relative standard deviations (R.S.D.) were calculated from the compound variance of the calibration standards assayed in duplicate within each series. Blank plasma samples spiked with known amounts of DDATHF were stored at −20°C for use as control samples and assayed in triplicate with each series. The analytical recovery of the assay and the between-run R.S.D. values were calculated from these control samples.

Rat study

Two male WAG/Rij rats were cannulated into the carotid artery. DDATHF (4 mg/kg) was administered intravenously into the tail vein. Blood (*ca.* 500 μl) was collected at 5 and 15 min and 1, 2, 4 and 6 h after administration of the drug. The animals were killed at 6 h or 24 h to obtain a large amount of blood (4–5 ml) by heart puncture. Plasma was separated immediately by centrifugation (5 min, 3000 *g*).

RESULTS AND DISCUSSION

Chromatography

Reversed-phase HPLC using C_{18} columns has been used successfully for the determination of tetrahydrofolates and analogues [5–8]. During the development of the present assay, several packing materials from different sources were tested. Marked differences in peak shape and column efficiency were found between the different brands of commercially available C_{18} packings. Nucleosil C_{18} was selected as the most suitable column packing for the determination of DDATHF as judged from the peak shape. Addition of an ion-pairing compound (1-octanesulphonic acid, PIC B-8) further improved the peak shape. The retention behaviour of DDATHF in this ion-pair HPLC system is very sensitive to changes in the amount of acetonitrile in the mobile phase (Table I). Under the present chromatographic conditions DDATHF is well separated from MTX (I.S.) and endogenous plasma compounds (Fig. 2).

TABLE I

RETENTION OF DDATHF AS A FUNCTION OF PERCENTAGE OF ACETONITRILE IN THE MOBILE PHASE

Acetonitrile concentration (%, v/v)	Capacity factor (k')
15	19.0
16	8.0
18	4.9
22	2.4

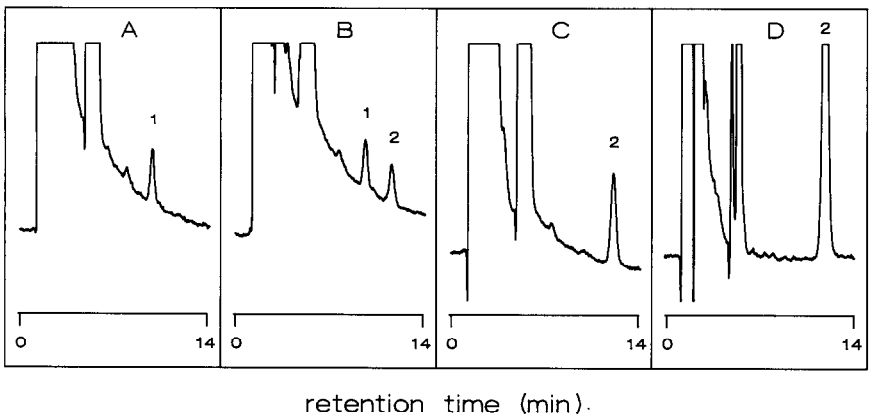

Fig. 2. Chromatograms of (A) a blank human plasma sample, (B) a human plasma sample spiked with 100 ng/ml DDATHF, (C) a rat plasma sample obtained 6 h and (D) a rat sample obtained 5 min after administration of 4 mg/kg DDATHF. No internal standard (MTX) was added to samples C and D to demonstrate the selectivity of the assay. Chromatograms A, B and C, 500-μl sample; D, 50-μl sample. Peaks: 1 = MTX; 2 = DDATHF. Sensitivity: 0.002 a.u.f.s.

Sample pretreatment

Several sample pretreatment procedures were investigated, including protein precipitation with perchloric acid and various types of solid-phase extractions. Cation-exchange solid-phase extractions on aromatic sulphonic acid material provided the most selective sample pretreatment procedure. The behaviours of DDATHF and MTX during the washing and elution steps were virtually identical. The absolute recovery of both compounds was 58.3 ± 4.6% (n = 4). The recovery was only marginally improved when more elution buffer was used. The pH of the eluate (pH 7.0) was adjusted to 3.5, which permits the injection of a large sample volume (100 μl) without introducing peak distortion. Typical chromatograms of plasma samples are depicted in Fig. 2, demonstrating the selectivity of this HPLC–UV method.

Validation

The minimum detectable concentration in plasma (signal-to-noise ratio = 3:1) was 10 ng/ml using a 500-μl sample. A limited sample volume

TABLE II

REPRODUCIBILITY OF THE ASSAY

Sample	Concentration (ng/ml)	%*RCR*	Within-run R.S.D. (%)	Between-run R.S.D. (%)	Recovery (%)
Standard	20	−3.8	5.2		
	50	0.3	1.7		
	100	−0.4	2.9		
	250	1.2	3.4		
Control	(150)		2.5	2.7	100.5
Standard	500	0.7	1.6		
	1000	−1.0	2.8		
	2000	0.2	1.3		
	5000	0.2	0.6		
Control	(1000)		2.0	2.6	105.2

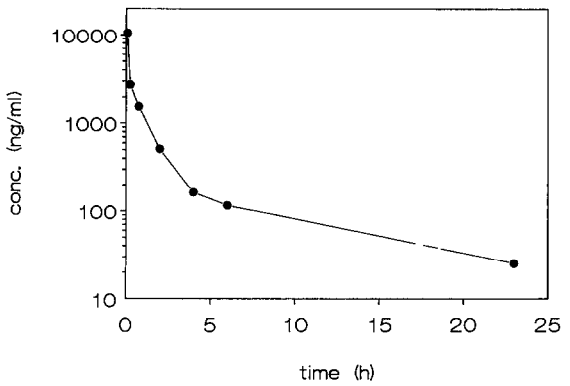

Fig. 3. Plasma concentration *vs.* time curve for DDATHF in the rat. Dose: 4 mg/kg intravenously.

(50 μl) was used for concentrations exceeding 250 ng/ml. As the R.S.D. was constant over the entire concentration range of interest and the calibration graphs were linear and passed through the origin, log–log transformation of the abscissa and ordinate is a valid method to perform weighted linear regression analysis [4]. The reproducibility was tested in ten analytical runs and the results are presented in Table II. It is important to perform the chromatographic analyses within 3 h after sample pretreatment or to store the eluates at 4°C for a maximum of 12 h, otherwise conversion of DDATHF in the eluate into a product with a retention time close to that of MTX occurs, which diminishes the analytical accuracy.

Applicability

The applicability of the assay was demonstrated in a study in rats (Fig. 3). The sensitivity of the method is sufficient to detect DDATHF in samples taken 24 h after the administration of 4 mg/kg. Apart from the DDATHF peak, no other peak emerged in the chromatograms.

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