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High-performance liquid chromatographic method for the analysis of 10-ethyl-10-deaza-aminopterin and metabolites in plasma

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ABSTRACT

10-Ethyl-10-deaza-aminopterin (10-EdAM) is a novel folic acid antimetabolite currently being tested in phase II clinical trials. We have developed an isocratic high-performance liquid chromatographic method for the quantification of 10-EdAM and metabolites in plasma. Solid-phase extraction was used for sample clean-up. Adequate accuracy was obtained without the use of an internal standard. Fluorometric detection with excitation at 243 nm and emission at 488 nm was used for accurate quantification of samples containing small amounts of drug or metabolites (2.0–4.0 n*M*, depending on the compound). Ultraviolet detection at 350 nm was only applicable for the analysis of plasma concentrations of 10-EdAM exceeding 50 n*M*. The usefulness of the assay was demonstrated by the results obtained in a pharmacokinetic study. The assay could separate the parent compound from seven identified and two unknown products.

INTRODUCTION

In the search for new and more effective antitumour drugs, several analogues of methotrexate (MTX) have been synthesized and tested for antitumour ef-

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ficacy. In preclinical studies 10-ethyl-10-deaza-aminopterin (10-EdAM) was found to be superior to a diverse array of other antifolate antimetabolites [1,2]. The interesting preclinical results [3,4] led to the clinical testing of 10-EdAM in a phase I trial [5]. The pharmacokinetics of the drug were defined by high-performance liquid chromatography (HPLC) with fluorometric detection [6]. This method, however, was based on gradient elution and achieved only moderate resolution between 10-EdAM and its 7-hydroxy metabolite. Kinahan et al. [6] mentioned other metabolites (e.g. polyglutamates of 10-EdAM), but no analytical data were reported.

This paper describes a simple, sensitive and accurate isocratic HPLC method, which shows good selectivity for 10-EdAM and its metabolites identified in the plasma of patients.

EXPERIMENTAL

Instrumentation and chromatography

UV spectra were recorded using a CARY 210 spectrophotometer (Varian, Zug, Switzerland). Fluorescence spectra were obtained using a SPF-500 RATIO spectrofluorometer (Aminco, MD, U.S.A.). The HPLC system consisted of a Spectroflow SF400 pump, an SF980 fluorescence detector, a SF757 variable-wavelength UV–VIS detector (Kratos, NJ, U.S.A.), an MSI 660 autosampler (Kontron, Zurich, Switzerland) with a 100-μl sample loop and a Model CR-3A integrator (Shimadzu, Kyoto, Japan). Samples were chromatographed on Chromsep glass columns (200 mm$\times$3 mm I.D.) packed with Chromspher C_{18} material, preceded by a guard column (10 mm$\times$3 mm I.D.) packed with pellicular reversed-phase material (Chrompack, Middelburg, The Netherlands). The mobile phase was methanol–10 m*M* ammonium formate with 1.0 g/l tetramethylammonium chloride (40:60, v/v). The pH was adjusted to 3.0 with hydrochloric acid, and the solution was degassed by vacuum. The flow-rate was maintained at 0.4 ml/min. For fluorometric detection the excitation wavelength was set at 243 nm, and the emission was monitored at 488 nm using a band-pass filter (bandwidth 11 nm). The response time was set at 5 s. Solid-phase extraction was performed on SPE C_{18} (1 ml) columns (J.T. Baker, Phillipsburg, NJ, U.S.A.). The columns were preconditioned according to the manufacturer's instructions.

Solvents and reagents

10-EdAM was obtained in vials containing 50 mg as the sodium salt (Ciba Geigy, Basle, Switzerland) and was used as such. 10-Ethyl-10-deaza-aminopteroic acid·HBr (10-EdAMpta) was a generous gift from Dr. W. Riess and Dr. L. Maier (Ciba Geigy). L-Glu-γ-L-Glu and L-Glu-γ-L-Glu-γ-L-Glu were synthesized in our institute by Dr. L. Vernie on a 9500/AT peptide synthesizer (Biosearch, San Rafael, CA, U.S.A.) using standard protocols. Ammonium

formate was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were purchased from E. Merck (Darmstadt, F.R.G.). Deionized water purified by the Millipore-Q system (Waters Assoc., Milford, MA, U.S.A.) was used throughout.

Sample preparation

A 500-μl volume of plasma was mixed with 500 μl of 0.15 *M* phosphate buffer (pH 6.5) and transferred to the top of a preconditioned SPE C_{18} column. The column was rinsed twice with 1 ml of 0.15 *M* phosphate buffer (pH 6.5) followed by 0.2 ml of water. The column was dried and elution was carried out with 2 ml of methanol. The eluate was evaporated under nitrogen (37°C) and the residue was dissolved in 200 μl of 10 m*M* ammonium formate buffer (pH 3.0). An aliquot of 100 μl was injected into the chromatographic system.

Synthesis of the metabolites

7-Hydroxy-10-EdAM was synthesized as described by Cairnes and Evans [7]. 10-EdAM di- and triglutamate were synthesized as follows. A 40-mg (130 μmol) amount of 10-EdAMpta in 1000 μl of ice-cold dimethylformamide (DMF) was mixed with 16 μl of triethylamine and 15 μl (116 μmol) of isobutyl chloroformate. The mixture was kept at room temperature for 15 min. Next, 32 mg of L-Glu-γ-L-Glu (116 μmol) or 50 mg of L-Glu-γ-L-Glu-γ-L-Glu (116 μmol) dissolved in 500 μl of DMF were added. Following overnight incubation, the solvent was removed by vacuum. The reaction product was purified by semi-preparative HPLC using a Spherisorb ODS2 column (100 mm×10 mm I.D.) (Phase Separations, Queensferry, U.K.) eluted with acetonitrile–0.05 *M* phosphate buffer (pH 7.0) (25:75, v/v). The fraction containing the polyglutamate was lyophilized, and the residue was dissolved in a small amount of water. Hydrochloric acid (5 *M*) was added until the pH was 3.0, and crystals were allowed to form overnight at 4°C.

All structures were confirmed by field desorption mass spectrometry (FD-MS), UV–VIS and fluorescence spectrophotometry. All synthesized compounds were virtually homogeneous as judged by the HPLC analysis. FD-MS of 7-OH-10-EdAM revealed two ions at *m/z* 484 (free acid) and *m/z* 506 (mono sodium salt). The di- and triglutamate metabolites showed ions at *m/z* 597 and *m/z* 726, respectively. Substantial amounts were present as mono-, di- or trisodium and/or potassium salt.

The absorption spectra and excitation spectra in 10 *M* ammonium formate buffer (pH 3.0), with emission monitored at 488 nm, of 10-EdAM, 10-EdAMpta, 10-EdAM di- and triglutamate showed identical profiles (excitation maximum at 350 nm). For calibration purposes we assumed that the relative molar fluorescence intensities of these compounds were independent of the number of glutamic acid residues present. The purities of the compounds calculated relative to 10-EdAMpta (which was assumed to be more than 99%

pure) were: 10-EdAM, 96.1%; 10-EdAM diglutamate, 48.6%; and 10-EdAM-triglutamate, 47.7%. The purity of the preparation of purified 7-OH-10-EdAM was determined indirectly. A known amount of 10-EdAM was incubated with partially purified aldehyde oxidase [8]. Conversion of 10-EdAM into 7-OH-10-EdAM was assumed to be quantitative as no side-products were detected. The peak area of the 7-hydroxy metabolite formed was calibrated on the decrease of the concentration of 10-EdAM. From these "standards" the purity of the purified 7-OH-10-EdAM was calculated to be 50.3%. 7-OH-10-EdAMpta, 7-OH-10-EdAM di- and triglutamate were synthesized by incubation of their respective parent compounds with partially purified aldehyde oxidase. They were not purified or characterized, but the peak appearing in the chromatogram was assumed to represent the 7-OH metabolite. No side-products were detected.

RESULTS AND DISCUSSION

Optimization of the chromatographic separation

The HPLC system described previously [6] gives a poor separation between the parent drug and its 7-hydroxy metabolite. Changing the solvent modifier solution had little effect if the pH of the mobile phase was kept at 7.0. The glutamic acid part of the molecule is deprotonated at this pH, resulting in relatively little retention of and selectivity between the two compounds. This applied to an even greater extent to the polyglutamates. At acidic pH (pH 3) the retention of both compounds had increased, and the selectivity could effectively be influenced by the choice of the modifier solution (Fig. 1). It was found that both methanol and tetrahydrofuran were equally effective in sepa-

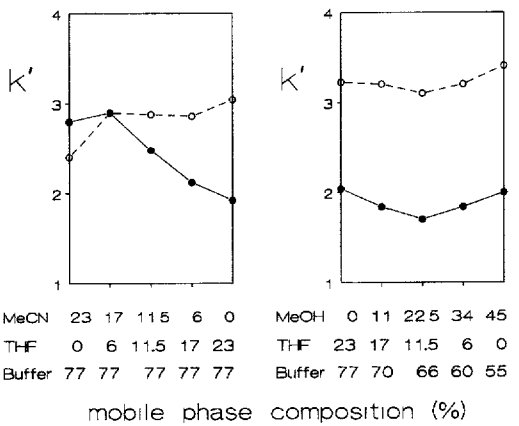

Fig. 1. Retention of (●) 10-EdAM and (○) its 7-hydroxy metabolite, as a function of mobile phase composition. MeCN = acetonitrile; MeOH = methanol; THF = tetrahydrofuran; buffer = 10 m*M* ammonium formate buffer (pH 3.0).

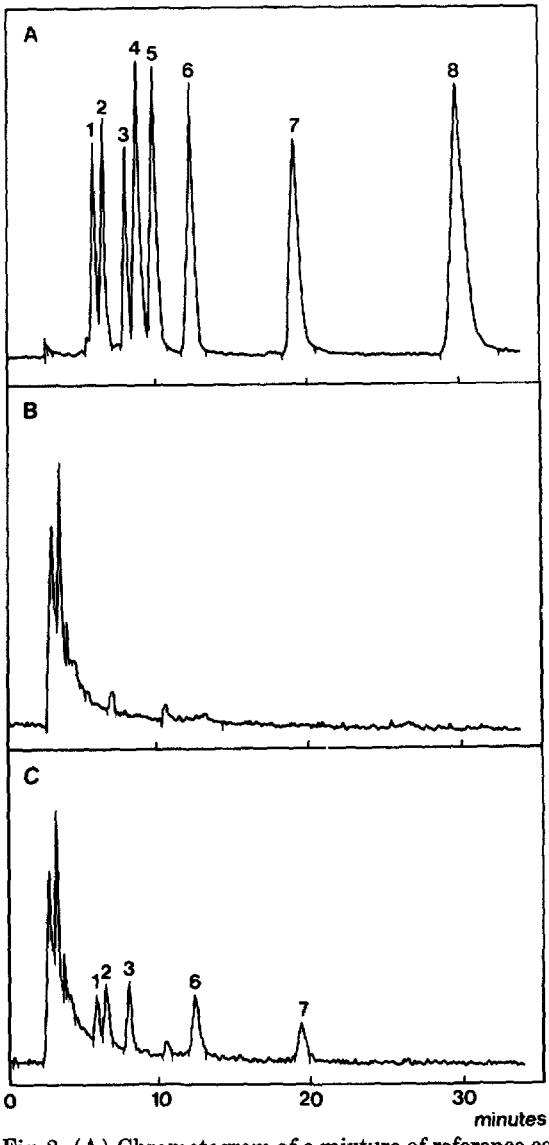

Fig. 2. (A) Chromatogram of a mixture of reference compounds. Chromatographic conditions as in text; fluorescence excitation at 243 nm, emission at 488 nm. Peaks: 1 = 10-EdAM triglutamate; 2 = 10-EdAM diglutamate; 3 = 10-EdAM; 4 = 7-hydroxy-10-EdAM triglutamate; 5 = 7-hydroxy-10-EdAM diglutamate; 6 = 7-hydroxy-10-EdAM; 7 = 10-EdAMpta; 8 = 7-hydroxy-10-EdAMpta. (B) Chromatogram of a blank plasma sample. (C) Chromatogram of the same plasma spiked with reference compounds (ca. 10 n*M* each).

TABLE I

CHARACTERISTICS OF THE CHROMATOGRAPHIC SYSTEM

A set of aqueous standards containing the reference compounds in the concentration range 7–1000 n*M* were analysed on three consecutive days. The slope (*x*-coefficient) and intercept of the calibration curves were calculated by linear regression. Correlation coefficients were always better then 0.99. The detection limits were given as the amount injected into the chromatographic system (signal-to-noise ratio 5).

Compound	Slope (mean ± S.D.)	Intercept (mean ± S.D.)	Detection limit (pmol)
10-EdAM triglutamate	12.3 ± 0.4	−67.4 ± 25.5	0.5
10-EdAM diglutamate	11.2 ± 1.0	−1.4 ± 51.5	0.5
10-EdAM	11.3 ± 0.6	+8.7 ± 21.5	0.5
10-EdAMpta	11.2 ± 0.1	−10.5 ± 21.6	1.0
7-OH-10-EdAM	26.8 ± 0.2	−5.4 ± 74.0	0.3

TABLE II

RECOVERIES OF 10-EdAM AND OTHER REFERENCE STANDARDS

Human blank plasma was spiked with compounds available as reference standards. The recovery was recorded relative to an untreated aqueous standard.

Compound	Concentration (n*M*)	Recovery (%)	Within-day coefficient of variation (%)	Limit of determination (n*M*)
10-EdAM triglutamate	9.7	72.8	17.8	2.9
	97	61.9	3.2	
	970	65.7	3.6	
10-EdAM diglutamate	10.8	87.1	12.8	2.6
	108	83.4	3.5	
	1080	87.2	2.3	
10-EdAM	9.6	100.9	6.5	2.0
	96	92.0	2.3	
	960	96.0	2.0	
	9600	96.6	1.9	
10-EdAMpta	11.0	94.4	3.5	4.0
	110	95.1	2.4	
	1100	99.9	1.1	
7-OH-10-EdAM	7.2	58.5	12.0	2.0
	72	58.4	6.8	
	720	56.1	5.7	

rating the compounds. The addition of a tetraalkylammonium salt diminished peak tailing. Fig. 2A shows the chromatographic separation of the compounds of interest. The resolution between 10-EdAM and 7-OH-10-EdAM digluta-

mate was sufficient to permit the detection of 5 n*M* 7-OH-10-EdAM diglutamate in the presence of 30 000 n*M* 10-EdAM.

Optimization of the detection

We investigated the applicability of UV detection for the analysis of 10-EdAM and its metabolites. The absorbance spectrum of 10-EdAM in a neutral solution revealed an absorption maximum near 252 nm and one at 360 nm with a lower specific extinction coefficient. At acidic pH the absorption maxima shifted to lower wavelengths with increased intensity. Despite the greater absorption at 242 nm, detection at 350 nm was preferred owing to the higher selectivity over other compounds. The presence of interfering compounds, however, still limits the use of the HPLC–UV method for samples containing relatively high concentrations (greater than 50 n*M*) of the drug.

Oxidized pteridine-containing compounds, such as 10-EdAM, show strong fluorescence properties, especially in solutions of neutral pH. At acidic pH the fluorescence diminishes. Fluorescence measurements depend strongly on the equipment used and particularly on the light source. The HPLC detector used in this study was provided with a deuterium lamp, which has its highest intensity in the low UV range. The optimal signal-to-noise ratio on this instrument was found to be near 243 nm. A 488-nm band-pass emission filter was used for optimal selectivity. In ca. thirty blank samples from cancer patients tested, no interfering peaks for 10-EdAM were present. In a few samples minor interferences (less than 5 n*M*) were present for 10-EdAM di- and triglutamate. The characteristics of the chromatographic system are presented in Table I.

Sample preparation

Solid-phase extraction was used for sample clean-up. The recoveries of the compounds were measured in samples spiked with known amounts and re-

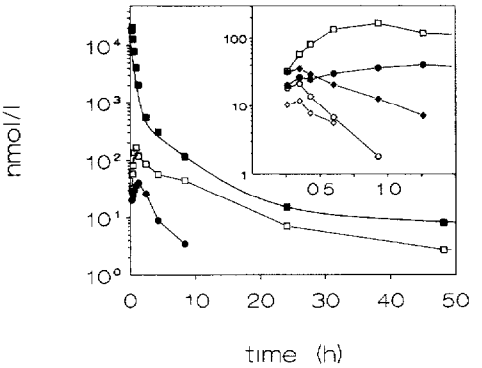

Fig. 3. Concentration–time curve of 10-EdAM and related products in plasma of a patient who received 80 mg/m^2 in an intravenous infusion over 15 min. The insert shows the same data with an expanded time-scale for the first 2 h. (■) 10-EdAM; (□) 7-hydroxy-10-EdAM; (●) 10-EdAM diglutamate; (○) 10-EdAMpta; (◆) unknown (u1); (◇) unknown (u2).

corded relative to an untreated aqueous standard sample. The recoveries showed no concentration dependence over the entire range tested (Table II). Plasma samples with concentrations of 10-EdAM over 1000 n*M* were diluted twenty-fold with ammonium formate buffer before injection into the chromatographic system. Since a good reproducibility was obtained, no internal standard was required.

Restriction

Salicylic acid and acetylsalicylic acid were found seriously to interfere with the quantification of 7-hydroxy-10-EdAM diglutamate, as the former two compounds have the same retention time and both show fluorescence.

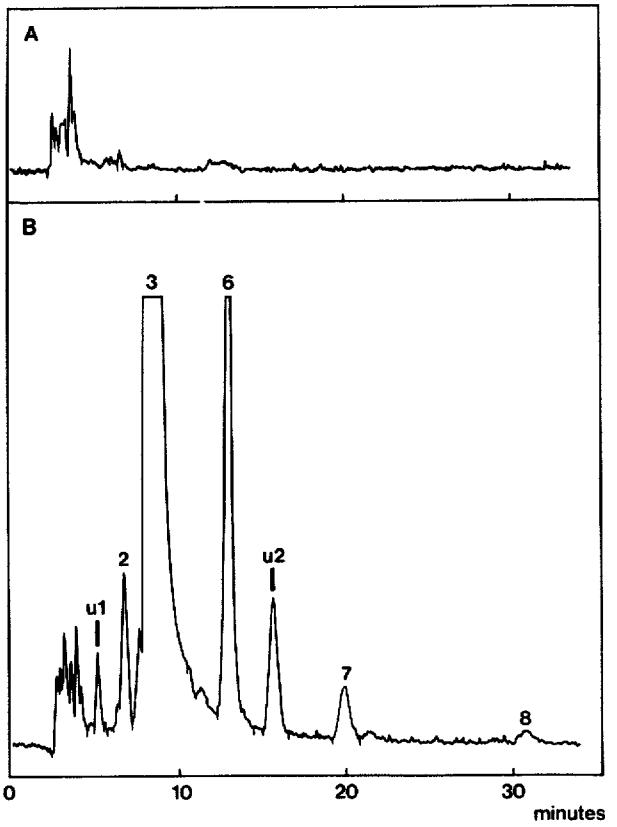

Fig. 4. (A) Chromatogram of a patient sample collected shortly before the administration of 10-EdAM. (B) Chromatogram of a patient sample collected 10 min after infusion of 10-EdAM. u1 and u2 are two unknown compounds present as impurities in the pharmaceutical product. For other peaks, see Fig. 2.

Plasma pharmacokinetics

The assay was used for studying the plasma pharmacokinetics in cancer patients. Fig. 3 depicts the concentration–time curves for the various compounds present in plasma of a patient. In addition to the peaks coeluting with our reference standards two more peaks were detected (Fig. 4). Analysis of the pharmaceutical product revealed that they represented impurities. It also contained some 10-EdAMpta (0.11%) and some 10-EdAM diglutamate (0.06%). Both the unknown products and 10-EdAMpta were rapidly cleared. The concentrations of 10-EdAM diglutamate and 7-OH-10-EdAM increased until 1 h after cessation of the infusion, which indicated metabolic formation. No detectable amounts of 7-OH-10-EdAM diglutamate or 10-EdAM triglutamate were present.

CONCLUSIONS

A simple, sensitive and accurate HPLC method is presented for the quantification of 10-EdAM, seven identified and two unknown products in plasma. Solid-phase extraction without the use of an internal standard gave sufficiently reproducible results.

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