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Note

Liquid chromatographic determination of an antineoplastic aziridinylbenzoquinone in human and murine serum

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In recent comparisons of the activity in vivo of a series of aziridinylbenzoquinones [1—3] one compound, 1,4-cyclohexadiene-1,4-dicarbamic acid 2,5-bis(1-aziridinyl)-3,6-dioxo diethyl ester (AZQ), demonstrated superior antitumor activity in rodents especially with respect to intracerebrally injected L1210 and P388 leukemias as well as several other tumor lines including ependymoblastoma, B-16 melanoma and C26 colon tumor. AZQ therefore represents a promising new lipid-soluble antineoplastic drug that may have significant activity against CNS tumors in humans. This drug is currently under Phase I investigation in patients with cancer at the National Cancer Institute, the Vermont Regional Cancer Center and elsewhere.

This paper presents the first report of a simple analytical method for determination of AZQ in serum samples that is sensitive to drug concentrations as low as 20 ng/ml. We describe a reversed-phase ion-pair isocratic chromatographic system that utilizes a variable-wavelength UV detector. The proposed method is also applicable for the analytical study of certain other aziridinylbenzoquinones which also have demonstrated significant antitumor activity in experimental animal tumor systems.

EXPERIMENTAL

Chromatographic apparatus

A Spectra Physics Model 8000 microprocessor-controlled high-performance liquid chromatograph equipped with a data system was used. The chromatograph was equipped with a Schoeffel Model 770 variable-wavelength UV detector set at 340 nm. The column was an Altex (Berkeley, CA, U.S.A.) Ultra-

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sphere I.P. 5-μm column (25 cm × 4.6 mm I.D.). A guard column (7 cm × 0.2 cm I.D.), packed with Co:Pell ODS, 25—37 μm (Whatman, Clifton, NJ, U.S.A.) was installed to protect the 5-μm column. Samples were injected onto the column through a 10-μl loop, using a manual injector.

Chemicals

High-performance liquid chromatography (HPLC) grade water and methanol were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). AZQ (NSC 182986) and appropriate diluents for clinical preparation were obtained from the Investigational Drug Branch, National Cancer Institute, National Institutes of Health. The AZQ analogs investigated were provided by Dr. John S. Driscoll (Laboratory of Medicinal Chemistry, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, MD, U.S.A.). PIC reagent A was purchased from Waters Assoc. (Milford, MA, U.S.A.).

Stock solutions of AZQ were made by solubilizing the drug in dimethylacetamide, and diluting with 0.1 *M* phosphate buffer, pH 6.5. Further dilutions were carried out in HPLC-grade water when necessary. Stock solutions of the water-soluble AZQ analogs were made by placing the compound in HPLC-grade water followed by sonication to break up residual particles. Analogs which were not water soluble were solubilized in dimethylsulfoxide and diluted to the desired concentration with HPLC-grade water. All solutions were filtered through a 0.45-μm Millipore filter before injection onto the column. The final concentration of AZQ was estimated using the extinction coefficient of the compound in methanol at 340 nm [1].

Drug administration and serum collection

Human. AZQ was administered in the wards and clinics of the Medical Center Hospital of Vermont and the University Health Center. The patients all had far advanced cancer unresponsive to conventional therapy. Written informed consent was obtained from all patients. AZQ was administered to patients over a 5-min period by injection into a running intravenous line containing normal saline. Doses up to 10 mg per square meter of body surface area with three or four patients per dose were examined in this Phase I study. Ten ml of whole blood were obtained from the arm opposite to that used for the drug injection. Serum was obtained by centrifugation and stored at —10°C for less than 48 h prior to analysis for AZQ content.

Mouse. AZQ was injected into the tail vein of female Swiss Webster mice (20—25 g) at a high dose (5—10 mg/kg) over a period of 10—20 sec. Blood samples were obtained by retro-orbital puncture at various times following injection. Serum samples were prepared by centrifugation of blood at 7000 *g* for 5 min in a bench top centrifuge and were either assayed immediately or frozen at —15°C for subsequent analysis (within 24 h).

Preparation of serum samples for analysis by HPLC

Serum samples were prepared for injection onto the column using Sep-Pak cartridges (Waters Assoc.). The use of the Sep-Paks affords both sample purification and drug concentration. Cartridges were prepared for use as described in the brochure supplied by Waters Assoc. A measured volume (0.5—4.0 ml) of

serum or stock drug solution was loaded onto the Sep-Pak, which was then washed with 1.0 ml HPLC-grade water containing PIC reagent A. AZQ was then eluted by washing the Sep-Pak with 3.0 ml HPLC-grade methanol containing PIC reagent A. The methanol was removed under nitrogen and the residue was taken up in a minimum volume (100—200 μl) of HPLC-grade water. The sample was vortexed, and then centrifuged to remove the insoluble material. The resulting solution was used directly for injection onto the column.

HPLC procedure

AZQ was eluted from the column using an isocratic mobile phase of water—methanol (65:35, v/v) containing 0.005 *M* tetrabutylammonium phosphate (PIC reagent A). A flow-rate of 1.0 ml/min was used. The concentration of AZQ in the eluate was measured by UV detection at 340 nm. Quantitative analysis was based on peak areas, and was computed using pre-set integration programs in the data system of the Spectra Physics HPLC instrument. Prior to sample analysis, the chromatograph was calibrated daily using AZQ solutions of known concentration.

RESULTS AND DISCUSSION

Fig. 1 illustrates the chromatogram obtained when AZQ was injected either as a stock solution (Fig. 1A) or as an extract of human serum (Fig. 1B). Drug elution occurred at a retention time that was well separated from the solvent front. A relatively minor increase or decrease in the percent of methanol in the described mobile phase permitted shortening or lengthening, respectively, of AZQ retention time as desired. The elution of AZQ using either isocratic or gradient systems of water and methanol without the PIC reagent A resulted in peaks which were very broad and poorly defined making accurate quantitation of peak area difficult. A standard calibration curve for AZQ was generated daily by plotting peak areas (as computed by the data system) against the concentration of the drug injected onto the column. The relationship was linear over a ten-fold concentration range, with an excellent correlation coefficient of 0.998 calculated using the least squares regression line. The correlation between measured peak height and drug concentration was also linear with an excellent correlation coefficient.

The minimum detectable level of AZQ was 7.0 ng injected, at a detector setting of 0.01 a.u.f.s. and a recorder attenuation of zero. The signal-to-noise ratio was 6 or less under these conditions.

The reproducibility of the described method was checked by sequentially injecting four samples of a stock AZQ solution (3.05 μg/ml) and then measuring retention times and areas of the eluted peaks. The standard error of the calculated concentration as peak area was less than 5% of the mean value (2.99 ± 0.14 μg/ml). The standard error of the mean retention time was less than 1% of the mean value (10.33 ± 0.06 min).

Several analogs of AZQ which had previously been shown to possess significant antitumor activity [1—3] were investigated using the described chromatographic procedure. The structures and retention times for these compounds

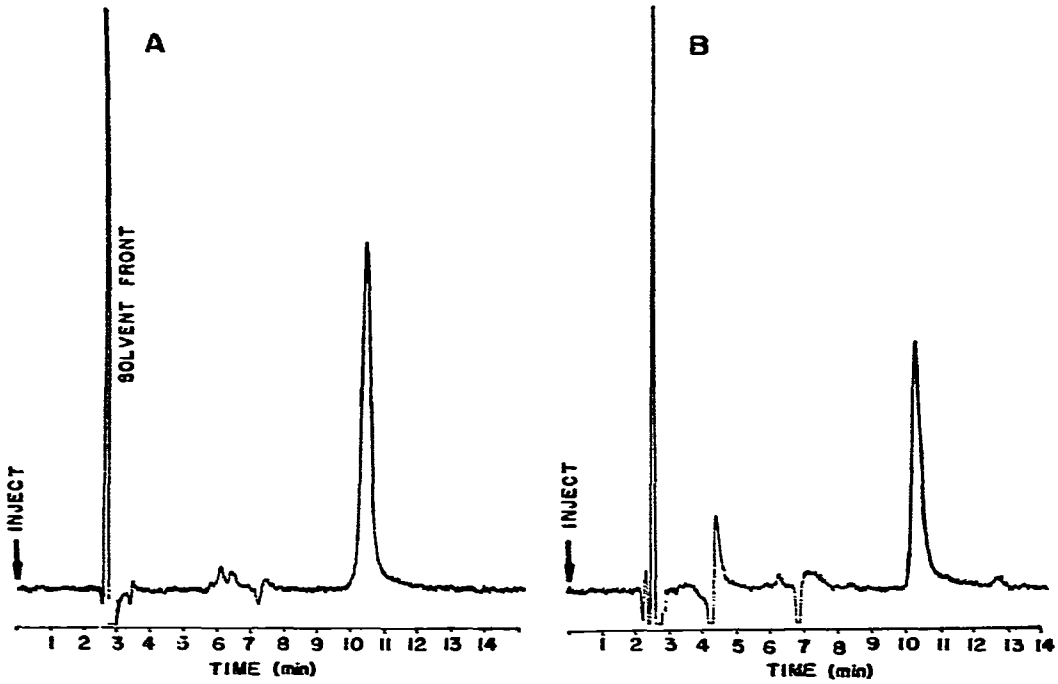


Fig. 1. Typical chromatograms of AZQ eluted from an Altex I.P. C_{18}-column. Conditions: column, 25 cm × 4.6 mm I.D.; mobile phase, water—methanol (65:35, v/v) with PIC A; flow-rate, 1.0 ml/min; column temperature, ambient; detector, Schoeffel 770 variable-wavelength UV (340 nm) at 0.1 a.u.f.s. (A) AZQ stock solution, 35 ng injected; (B) AZQ extract from human serum, the sample was drawn 5 min after the patient had received AZQ (10 mg/m²).

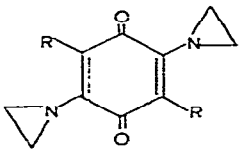
are shown in Table I. All of the alkyl-substituted analogs investigated showed a distinct peak, with retention times varying from 4.3 to 27.2 min. These retention times can be altered by minor changes in the percent of methanol in the mobile phase. Neither the piperidine nor the pyrrolidine analogs showed a peak within 30 min after injection. The piperazine-substituted compound, which also contained a substituted alkyl side-chain, showed a distinct peak at 4.8 min after injection. None of the analogs investigated interfered with the peak for AZQ. In addition, when the compounds were cochromatographed, each drug retained its individually determined retention time. The dimethylacetamide and phosphate buffer used to dissolve the AZQ did not interfere with the detection of the drug in this system.

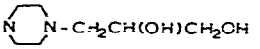
In order to determine the recovery of AZQ from plasma, samples of human plasma were spiked with known concentrations of AZQ, and then extracted using the Sep-Pak cartridges. Even when the plasma samples were spiked with relatively low concentrations of AZQ (e.g., 1—3 μg/ml), the recovery of the drug from the plasma was 90% or greater as calculated by the data system of the chromatograph. Recovery of AZQ from human serum samples was identical to that obtained in human plasma samples.

As can be seen in Fig. 2, administration of AZQ to humans results in a rapid elimination of this drug from serum. There appears to be an initial rapid phase of

TABLE I

RETENTION TIMES OF AZQ ANALOGS



R group	Retention time (min)
$NHCOOC_2H_5$ (AZQ)	10.3
NH_2	4.3
$NHCH_3$	6.9
$N(CH_3)_2$	27.2
$NHCH_2CH_2OH$	5.6
$NHCH_2CH(OH)CH_2OH$	4.8
	4.8
	>30.0
	>30.0

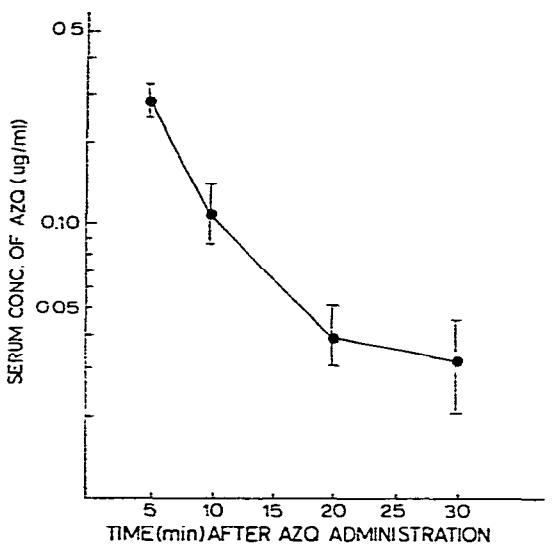


Fig. 2. Serum AZQ concentration in four patients given 10 mg/m² AZQ intravenously. Sample drug concentrations were determined by the described HPLC method. Data are presented as mean ± S.E.

drug distribution, followed by a rapid elimination phase with a half-life ($t_{1/4}$) of approximately 8 min. Serum drug levels were detectable through at least 30 min in patients who received 7 or 10 mg/m² AZQ. Drug levels in patients receiving less than 7.0 mg/m² AZQ were not always detectable at other than the 5-min time period. This phenomenon appears to be a function of the rapid

drug elimination kinetics and necessitates early and frequent serum drug level determinations if an accurate pharmacokinetic profile of this compound is to be done.

AZQ serum levels were also measured in Swiss Webster mice administered 10 mg/kg (30 mg/m^2) AZQ by tail vein injection. Our studies demonstrated that serum AZQ distribution and elimination phases were extremely rapid and closely resembled those observed in humans. Peak serum levels in the mice were much higher (2.0 μg/ml) than those determined in humans (0.3 μg/ml), an expected observation in view of the higher dose administered to mice and the shorter injection time. The serum $t_{1/2}$ in mice was estimated to be approximately 5 min. We currently are applying this method for analysis of AZQ to more extensive studies of the pharmacokinetics of this new drug in human patients and in experimental animals.

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