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COUNTER-CURRENT CHROMATOGRAPHY OF DIAZIQUONE BY A HORIZONTAL FLOW-THROUGH COIL PLANET CENTRIFUGE

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SUMMARY

Diaziquone is an active antitumor agent in animals that is unstable in aqueous solution. The chemical hydrolysis products of this antitumor agent have been identified and we have investigated the usefulness of the coil planet centrifuge in separating these products from the parent compound. We were able to separate the reactive diaziquone from the two reaction products using a solvent system containing dichloroethane, methanol, and sodium phosphate buffer. The identities of the products separated on the coil planet centrifuge were confirmed by thin-layer chromatography and mass spectrometry. This technique may be useful in separating metabolites contained in specimens obtained from diaziquone-treated patients.

INTRODUCTION

Diaziquone [2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone] belongs to a class of compounds which are active antitumor agents in animals. Because its structure suggests that it may be able to penetrate the central nervous system and therefore be effective against human central nervous system tumors, diaziquone was tested against intracerebral tumors. In addition to being effective against murine intraperitoneal L1210 leukemia, P388 leukemia, and B16 melanocarcinoma, diaziquone is effective against intracerebral L1210 and P388 leukemias and intracerebral ependymoblastoma[1]. Most recently, diaziquone is being assessed for activity in human malignancies[2].

Diaziquone contains two aziridine rings (Fig. 1) which open instantaneously at pH 1 to form the dialcohol (2 ROQ in Fig. 1)[3]. In weakly acidic solution (pH 3–5), diaziquone is hydrolyzed slowly so that the monoalcohol (1 ROQ in Fig. 1), an intermediate form, is present. These reaction products have been separated by high-performance liquid chromatography[3]. Since we are studying the human pharmacology of diaziquone, we wanted to use the horizontal flow-through coil planet centrifuge (HFCPC) to separate the reaction and metabolic products of diaziquone.

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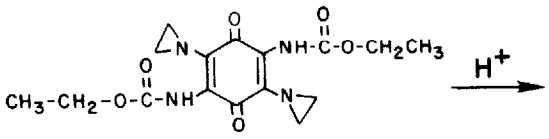
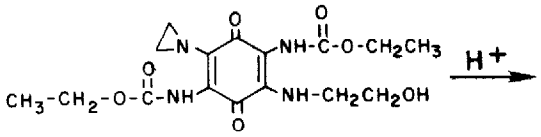


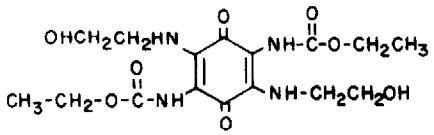
2,5-Diaziridinyl-3,6-bis (carboethoxyamino)-
1,4-benzoquinone

DIAZIQUONE



2-Aziridinyl-5-(2'-hydroxyethylamino)-
3,6-bis(carboethoxyamino)-1,4-benzoquinone

1 ROQ



2,5-di(2'-hydroxyethylamino)-
3,6-bis(carboethoxyamino)-1,4-benzoquinone

2 ROQ

Fig. 1. Structure of diaziquone and hydrolysis products 1 ROQ and 2 ROQ.

Others in our laboratory have been successful in separating other antitumor drugs and their reaction products using the HFCPC[4]. We concluded that this new non-destructive technique[5] would be particularly helpful in separating the very reactive diaziquone from its hydrolysis products.

MATERIALS AND METHODS

The coil planet centrifuge used was designed and constructed by Ito and consisted of 1000 helical turns of a 2.6-mm I.D. PTFE tubing with a total capacity of 270 ml[5]. The coiled column traversed a planetary motion about a central axis during chromatography, and a Milton-Roy minipump pumped solvents through the coil. An LKB Uvicord detector set at 280 nm and recorder were used, and fractions were collected by an LKB fraction collector.

The hydrolysis of diaziquone was done as follows: 4 mg of diaziquone were dissolved in 160 μl of dimethylacetamide and 1.44 ml of 10 m*M* citrate–phosphate buffer, pH 3, were added. This solution was allowed to stand at room temperature for 0, 6 or 24 h. The reaction was stopped by neutralizing with a drop of 1 *M* potassium

phosphate buffer, pH 7.5. Before injection into the coil, the sample was diluted with an equal volume of the two-phase system to be used in the separation.

The coil was filled with the stationary phase and then the sample was injected into the coil. The mobile phase was immediately pumped into the coil at 30 ml/h and the column rotation speed was brought up to 400 rpm. Separations required 16 h. At the end of the run, the fractions containing UV-absorbing material were pooled and evaporated to dryness. The residues were dissolved in a constant amount of chloroform–methanol (1:1) and 20 μl of each were applied to a thin-layer chromatography (TLC) plate (silica gel 60 F-254, with fluorescent indicator, E. Merck). The plate was developed in chloroform–methanol–ammonium hydroxide (20:5:1). Materials on the plate were located by UV-quenching and by nitrobenzylpyridine spray[6].

Four two-phase solvent systems were used in the HFCPC: (A) dichloroethane–methanol–10 m*M* sodium phosphate, pH 6.1 (3:3:1); (B) dichloroethane–methanol–10 m*M* sodium maleate, pH 6.1 (3:3:1); (C) chloroform–ethylene glycol (1:1); (D) *n*-hexane–ethyl acetate–nitromethane–methanol (8:2:2:3).

These solvent systems were mixed in a 2-l separatory funnel, and the phases were separated and filtered prior to use. Depending on the solvent system, either the upper or lower phase was chosen as the stationary phase, and the HFCPC was first filled with it. Following injection of the sample, the mobile phase (either upper or lower phase) was pumped through the HFCPC to elute the components. The choice of stationary and mobile phases also depends on the material being separated. Since diaziquone is so non-polar, in systems A and B we chose the lower phase to be mobile. However, in the non-aqueous systems, C and D, we chose the upper phase as the mobile phase.

Acetylation of the reaction products was accomplished by the addition of 0.3 ml of a 1:2 mixture of redistilled acetic anhydride and redistilled pyridine to the dry residues. After 3 h at room temperature, the solvents were evaporated by a stream of nitrogen.

Electron ionization mass spectrometry was performed on a VG Micromass 30F (VG Analytical, Altrincham, Great Britain) mass spectrometer operated under VG Datacomputer 2040 computer control. Source conditions were 200°C, 70 eV electron energy, 120 μA trap current, and 4 kV accelerating voltage. Samples were introduced to the source via the direct probe.

RESULTS AND DISCUSSION

Using solvent system A, we were able to separate the hydrolysis products of diaziquone on HFCPC with the lower phase as the mobile phase. The timed hydrolysis of diaziquone showed production of both 1 ROQ and 2 ROQ as products (Fig. 2). Three separate chromatographic runs show each time point of diaziquone hydrolysis. The 0-h control shows that diaziquone elutes from the coil at approximately fraction number 73, and no hydrolysis products are present. After 6 h at pH 3, diaziquone has reacted sufficiently to show the 1 ROQ and 2 ROQ as products. 2 ROQ is the most polar product and is eluted from the coil about fraction number 180, much later than 1 ROQ and parent compound. By 24 h, 2 ROQ is the major component of the reaction mixture.

Solvent system B also separated diaziquone and its hydrolysis products effec-

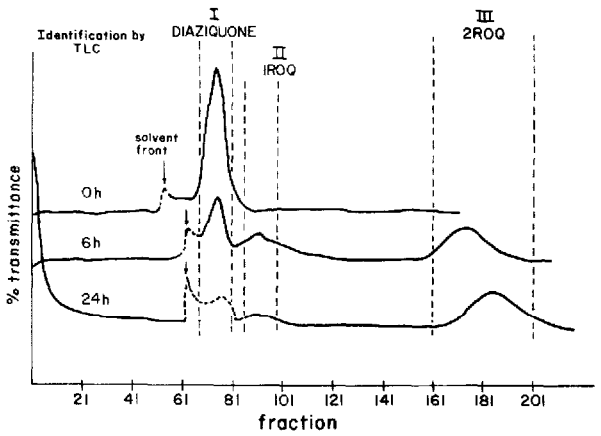


Fig. 2. Chromatograms of diaziquone and reaction products from the coil planet centrifuge. Samples were prepared and injected into the HFCPC as described in Materials and methods. The solvent system used was dichloroethane–methanol–10 m*M* sodium phosphate, pH 6.1 (3:3:1) (system A) with the lower phase as mobile phase. Vertical dotted lines indicate fractions that were pooled and dried for TLC (see Materials and methods). R_F and nitrobenzylpyridine spray were used to identify the materials on the plate. No diaziquone or products were detected in solvent-front fractions.

tively (Fig. 3). This system resulted in better resolution between diaziquone and 1 ROQ than did system A. The 2 ROQ product eluted from the coil at about fraction number 200, later than in system A, and in the 6-h reaction mixture the 2 ROQ did not elute from the coil.

Products I and III from the 6-h run in system A (Fig. 2) were analyzed by mass spectrometry as described in Materials and methods. The mass spectral analysis of product I indicated its molecular weight (*m/z* 364) as well as the successive loss of one (*m/z* 318) and two (*m/z* 272) molecules of ethanol (Table I). The mass spectrum of

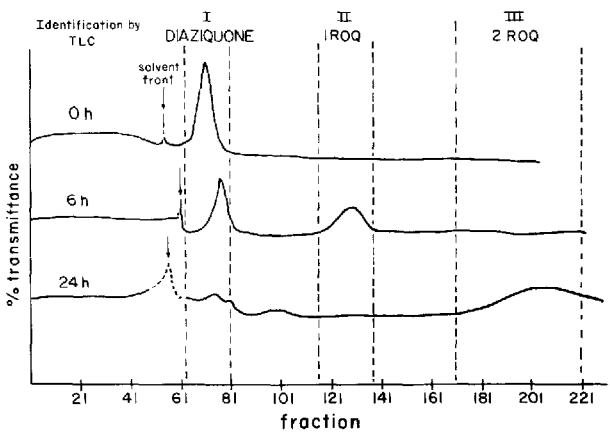


Fig. 3. Chromatograms of diaziquone and reaction products from the coil planet centrifuge. Details are the same as Fig. 2 except that the solvent system used was dichloroethane–methanol–10 m*M* sodium maleate, pH 6.1 (3:3:1) (system B) with the lower phase as mobile phase.

TABLE I

MASS SPECTRA OF I AND ACETYLATED III

Mass (relative intensity)		*Probable assignment*
I	*IIIa*	
364 (11)	484 (27)	M^{+}
318 (15)	438 (8)	$M - C_2H_5OH$
–	411 (17)	$M - CH_2OCOCH_3$
272 (11)	392 (7)	$M - 2\,C_2H_5OH$

product I was identical to the spectrum of pure diaziquone (spectrum not shown). Mass spectral analysis of acetylated product III indicated parent molecular weight (*m/z* 484) (Table I), the loss of one (*m/z* 438) and two (*m/z* 392) ethanol molecules, and the loss of the group (CH_2OCOCH_3) at *m/z* 411, characteristic of an acetate of a primary alcohol. The spectrum of acetylated product III is identical to the published spectrum[7].

We were unable to obtain a mass spectrum of product II (1 ROQ); however by TLC product II migrates as a UV-quenching material with an R_F between diaziquone and 2 ROQ. Product II gives a positive reaction with nitrobenzylpyridine spray, indicating that the material still has one aziridine ring intact.

Two non-aqueous solvent systems, C and D, were tried in the HFCPC to separate diaziquone and its hydrolysis products. System C (with upper phase as mobile phase) wax difficult to use, since ethylene glycol does not evaporate readily under vacuum. This system was also unsatisfactory because diaziquone alone injected into the coil resulted in three peaks of UV-absorbing material. The other non-aqueous solvent system, system D, has been used by Becker *et al.*[8] to separate epoxytriesters from plants. With either upper or lower phase as mobile phase, this system did not separate diaziquone from its hydrolysis products.

Our results show that diaziquone and its hydrolysis products can be separated by the coil planet centrifuge with the dichloroethane–methanol–10 m*M* sodium phosphate or maleate, pH 6.1 (3:3:1) solvent systems. This method for separating the very reactive substance diaziquone from reaction products is useful and important for the study of animal and clinical pharmacology of diaziquone.

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