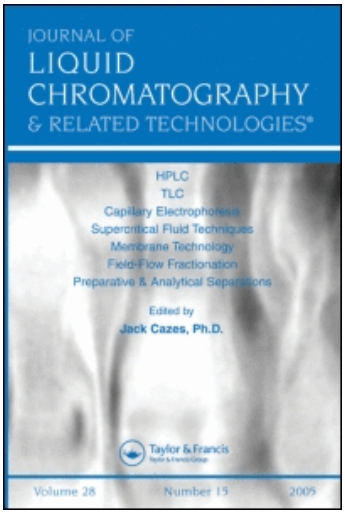

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AN HPLC METHOD FOR THE QUANTITATIVE DETERMINATION OF 1,4-DIHYDROXY-5,8 BIS [[2-[(2-HYDROXYETHYL) AMINO] ETHYL] AMINO] 9,10 - ANTHRACENEDIONE (DHAQ, LEDERLE LABS CL232 315, NCS 301739) IN SERUM

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ABSTRACT

An HPLC method using reverse phase chromatography is presented for the quantitative determination of 1,4-Dihydroxy-5,8 Bis [[2-[(2-Hydroxyethyl) amino] ethyl] amino] 9,10 - anthracenedione in serum and urine at levels which are expected to be found in clinical trials of this drug.

INTRODUCTION

In recent years there has been considerable interest focused on the Anthracycline antibiotics as potentially important anti-tumor agents. In particular, Doxirubicin and Daunorubicin have proven to be clinically useful in a wide range of human tumors (1). However, the cumulative dose dependent cardiotoxicity of these drugs have been limiting factors in their usefulness (2, 3). Two Anthracenedione derivatives 1,4-Dihydroxy 5,8 Bis [[2-[(2-Hydroxyethyl) amino] ethyl] amino] 9,10 Anthracenedione (DHAQ, CL232 315, NCS 301739) and the anhydroxy form 1,4-Bis [[2-[(2-Hydroxyethyl) amino] ethyl] amino] 9,10 anthracenedione (HAQ, NCS 287513) have

recently been reported to be active against several tumor systems (4, 5, 6) and DHAQ is currently being used in Phase I clinical trials. Unfortunately, there is at present no published method for the quantitative determination of these drugs at the levels anticipated in clinical samples. This paper reports an HPLC method for the analysis of DHAQ.

CHROMATOGRAPHIC CONDITIONS

Chromatography was performed using a μBondapak C18 column (3.9 mm I.D. x 30 cm) fitted with a 3.9 mm x 4 cm guard column of C18 corasil (Waters Associates). A model 6000A pump was used for solvent delivery, sampling was performed automatically with a WISP 710A autoinjector and detection was performed at 546 nm using a model 440 UV/visable spectrometer (Waters Associates). Peak area integration was accomplished with a Shimadzo integrator (Courtesy of Waters Associates). The mobile phase consists of 4.4 M ammonium formate (pH 4.3): acetonitrile:H_2O in a ratio 2:1:1. The ammonium formate solution was prepared by either of the following methods: a) 100 grams of dessicated ammonium formate and 25 ml of 88% formic acid (Fisher Scientific) were mixed and diluted to 500 ml with distilled deionized water; or b) 96 ml of formic acid was titrated with ammonia to pH 4.3 and adjusted to 500 ml with H_2O. All solvents were vacuum filtered through a .45 μ filter (Milipore) and degassed by sonication for 30 minutes prior to use.

SAMPLE PREPARATION

For each ml of plasma or serum 2 ml of a solution of $CHCl_3$: CH_3OH:6N HCl in a ration of 3.8:1.9:0.3 is added and mixed vigorously.

After centrifugation for 5 minutes at 1000 x g the upper phase is carefully removed discarding the lower phase and protein interface. The aqueous phase is then made basic by the addition of 200 µl of 50% ammonium hydroxide. Two ml of $CHCl_3$ is then added and after vigorous mixing and centrifugation for 5 minutes at 1000 x g the upper phase and remaining protein interface is removed and discarded. The lower organic phase is then taken to dryness under a stream of air and redissolved in 100 µl of the chromatographic solvent. All glassware is silanized prior to use with surfasil (Pierce Chemical Company) in order to prevent drug binding to the glass surfaces.

RESULTS AND DISCUSSION

DHAQ is a blue compound having several absorption maxima, the major bands being located at 662, 611 and 246 nm with molar extinction coefficients in the chromatographic solvent of 24, 19, and 40 x 10^3 respectively (Figure 1). Though it would be expected that maximum sensitivity could be achieved by monitoring UV absorption at 246 nm the presence of other UV absorbing material extracted from the serum eluting in the region of the drug negates any advantage due to the increased molar absorbtivity. This problem is obviated by measuring absorption in the visable region. Therefore, the eluent is monitored at 546 nm which is the highest filter available for our system. A typical chromatographic run on the drug is shown in Figure 2A. As can be seen there are several additional compounds present which also absorb at 546 nm though they amount to less than one percent of the total absorbing material in freshly prepared

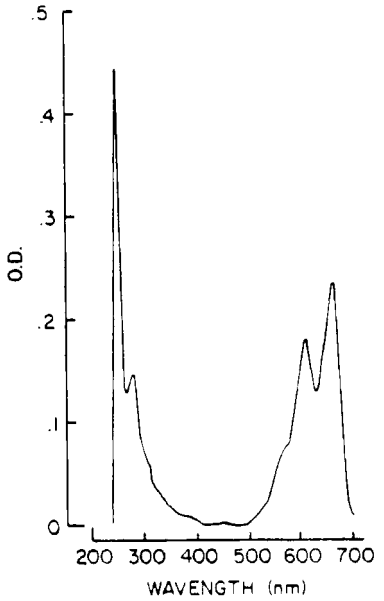

Figure 1 - The spectrum of 5 µg/ml of DHAQ in chromatographic solvent.

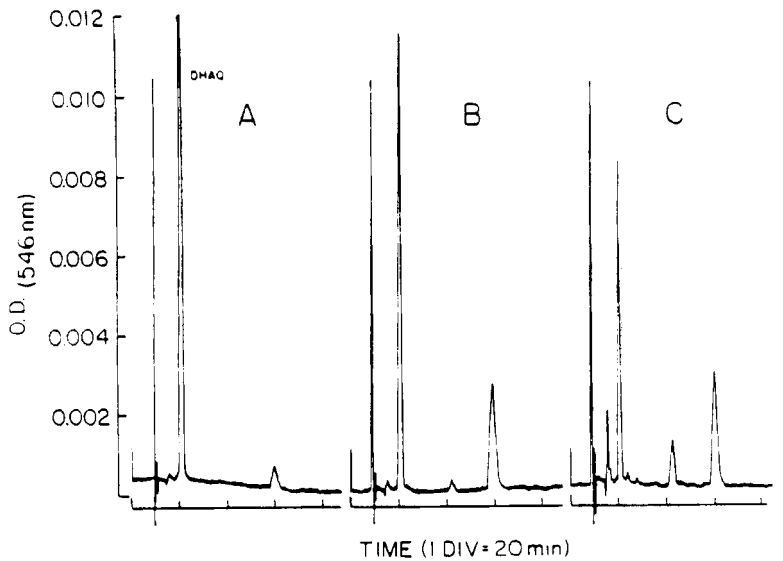

Figure 2 - The results of chromatographic separation of A) freshly prepared DHAQ B) DHAQ (40 µg/ml) allowed to stand one month C) DHAQ (40 µg/ml) allowed to stand seven months. Each chromatogram was obtained by applying 250 ng of total material to the column.

solution. However, the compound is not stable for extended periods in dilute solution (Figure 2B and C).

Several solvent systems were tried in an effort to determine the optimal separation conditions with C18 reverse phase columns. Elution of the drug could be achieved with iso-butanol but neither acetonitrile/water nor methanol/water in any proportion was capable of eluting the drug from either C18 or CN μBondapak (Waters Associates) columns. The addition of an anionic species could lower the K', but both phosphate and acetate produced peaks with marked tailing even in molar quantities. Formate ion proved to be the most effective in both reducing peak tailing and K'.

In order to assure a reasonable column life it is necessary to remove the drug from its biological matrix prior to chromatography. It was found that the drug binds non-specifically to proteins and that precipitation methods such as TCA or PCA lead to very poor recoveries. This problem was allievated somewhat by using an acid:methanol:chloroform mix to denature the protein which collects at the interface between the organic and aqueous layers. Advantage could then be taken of the difference in partition coefficients between the free base and its salt by using an additional chloroform extraction from the aqueous phase made basic with ammonium hydroxide. With this method of extraction efficiencies between 90 and 100% are routinely obtained when extracting from water, approximately 80-100% from urine and between 60 and 80% from serum. The lowered efficiency of serum and urine extraction is related to protein content. Because of the variability of

extraction, cresyl violet (Eastman) is routinely added as an internal standard prior to sample extraction. A typical chromatogram obtained from an extract of serum containing 250 ng of DHAQ and 300 ng of cresyl violet is shown in Figure 3. A plot of the ratio of area under the drug peak to area under the internal standard peak for serum and urine spiked with various drug concentrations over the range expected to be found in clinical trials of this drug is shown in Figure 4. The results obtained thus far indicate that the assay is reproducible within 10%.

In summary the extraction and chromatographic procedure presented in this report is capable of detecting and quantitating

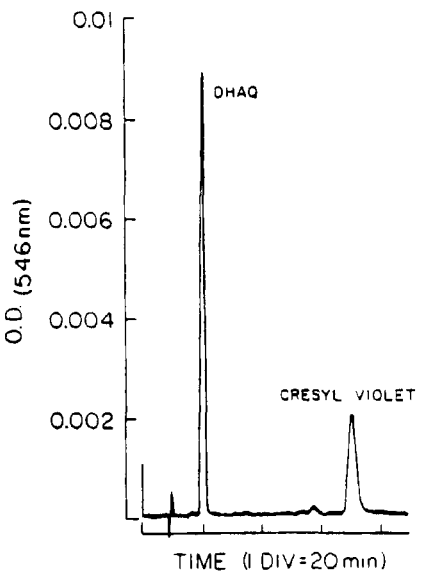

Figure 3 - A typical chromatogram obtained by spiking 1 ml of human serum with 250 ng of DHAQ and 300 ng of cresyl violet. Sample preparation is described in the text.

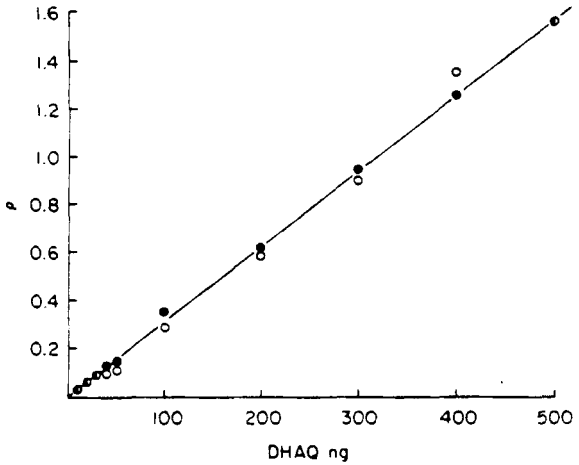

Figure 4 - A plot of the ratios (ρ) of the area under the curve of DHAQ to cresyl violet (internal standard) as a function of increasing drug concentration in human serum (●) and urine (○). Ordinate is amount of DHAQ applied to column. Results shown are the average of duplicate determinations.

approximately 10 ng of the anthracenedione DHAQ (CL232 315, Lederle Laboratories) when applied to a C18 reverse phase column. Preliminary results of patients entered into Phase I clinical trials indicate that this is the range of sensitivity required.

ACKNOWLEDGMENT

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