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CLINICAL ANALYSIS FOR THE ANTI-NEOPLASTIC AGENT 1,4-DIHYDROXY-5,8-BIS{{2-[(2-HYDROXYETHYL)AMINO]ETHYL}-AMINO} 9,10-ANTHRACENEDIONE DIHYDROCHLORIDE (NSC 301739) IN PLASMA

APPLICATION OF TEMPERATURE CONTROL TO PROVIDE SELECTIVITY IN PAIRED-ION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An analytical method is described which permits monitoring of plasma levels of the anti-tumor agent 1,4-dihydroxy-5,8-bis{{2-[(2-hydroxyethyl)amino]ethyl}amino}9,10-anthracenedione dihydrochloride (DHAD) following its intravenous administration to cancer patients. The drug cannot be efficiently extracted from plasma into water-immiscible solvents, but is effectively separated from the biological matrix by retention on hydrophobic XAD-2 beads packed in a disposable glass cartridge. DHAD is subsequently selectively eluted from this column and then analyzed by reversed-phase partition chromatography with spectrophotometric detection of the analyte. Resolution of overlapping bands during high-performance liquid chromatographic separation was achieved by systematic optimization of mobile phase, ion-pairing agent and temperature. A possible explanation for the observed selectivity provided by temperature adjustment is offered. Plasma levels in the range of 75—3000 ng of DHAD per ml (7.5—300 ng applied to the column) can be analyzed with a precision of $< \pm 10\%$. Total recovery of drug from plasma is ca. 95%.

INTRODUCTION

The anthracenedione, 1,4-dihydroxy-5,8-bis{{2-[(2-hydroxyethyl)amino]-ethyl}amino}9,10-anthracenedione dihydrochloride (NSC 301739; DHAD) is an analog of adriamycin that has shown significant antitumor activity in several animal tumor systems [1—3]. Preliminary studies indicate that DHAD is less

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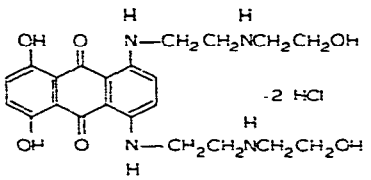


cardiotoxic than adriamycin in the rat cardiotoxicity model [4], thus, this drug represents a candidate agent with potential effectiveness against solid tumors and leukemia without the dose limiting cardiotoxicity associated with adriamycin. Initial clinical studies (Phase I) with DHAD indicate leukopenia and thrombocytopenia are dose limiting and suggest more rigorous clinical studies (Phase II) be carried out at an initial dose of 12 mg/m^2 repeated at 21—28-day intervals [5] to evaluate the therapeutic value of the drug. As part of these studies, the distribution and elimination of drug must be defined, necessitating drug level monitoring in plasma.

The purpose of the present study was to develop a clinically-useful assay for DHAD. Since the initiation of this work two papers concerned at least in part with the analysis of DHAD have appeared [6,7]. In the first, the authors describe a gradient elution HPLC procedure for separating nine aminoanthraquinone analogs. Temperature changes were also used to enhance resolution. While these authors proposed that the method was suitable for analysis in biological fluids, they did not address the problem of interference by biological compounds. Additionally, the utilization of a system which is neither isocratic nor isothermal would not be particularly suitable for routine clinical use.

In the report of Ostroy and Gams [7], which appeared subsequent to the completion of our own work, a method is described which utilized solvent extraction of DHAD and isocratic separation using high-performance liquid chromatography (HPLC). The major criticisms of the method are (a) the authors' failure to consider the instability of DHAD in plasma in designing the final analytical method and (b) the relatively inefficient recovery of DHAD and imprecision observed as a result of the tedious procedure for extraction of the drug from biological fluids.

Since our own studies have unequivocally demonstrated that DHAD is rapidly degraded in plasma, the validity of the method of Ostroy and Gams [7] is in question.

The present paper describes a method which appears to be suitable for clinical analysis of DHAD in plasma. It involves stabilization of drug in the biological matrix, separation of drug from biological fluid by retention on XAD-2 beads, elution from the resin and subsequent HPLC analysis with spectrophotometric detection.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump, a Model 710A Waters Intelligent Sample Processor (WISP) and a Model 440 Absorbance Detector. Peak areas were obtained mechanically with a Model 1810L10 Polar Planimeter (Dietzgen, Des

Plaines, IL, U.S.A.) and electronically with a Varian Assoc. (Palo Alto, CA, U.S.A.) Model 111-C Chromatography Data System (CDS 111-C) interfaced with the 10-V output of the Model 440 Absorbance Detector. The WISP initiated the CDS 111-C integration cycle upon automatic injection, and was programmed to make triplicate 100-μl injections (at slow syringe speed) with a 20-min run time per injection. The CDS 111-C was programmed for an initial peak width of 20 sec and a stop time of 18 min. For the developmental work, a Waters Assoc. Model U6K injector was used in place of the WISP and a Waters Assoc. Model 660 Solvent Programmer was utilized for mobile phase optimization. Separation was obtained with a Waters Assoc. μBondapak C_{18} column (30 cm × 3.9 mm I.D.; 10 μm particle size) and column eluent was monitored at 254 nm at a sensitivity of 1.0 a.u.f.s.

Materials

DHAD was supplied as the dihydrochloride salt by the National Cancer Institute and was used as obtained. The sodium salts of butane-, pentane-, hexane- and octanesulfonic acid (Eastman Kodak, Rochester, NY, U.S.A.) and ammonium dihydrogen phosphate AR (Matheson Coleman and Bell, Norwood, OH, U.S.A.) were used as obtained. Methanol (Fisher, Springfield, NJ, U.S.A.) and 2-propanol (J.T. Baker, Phillipsburg, NJ, U.S.A.) were HPLC grade. Amberlite XAD-2 resin (20—50 mesh) (Rohm and Haas, Philadelphia, PA, U.S.A.) was reduced to 100—200 mesh by dry milling the resin in a Micro Mill (Lab Apparatus, Cleveland, OH, U.S.A.). The pulverized resin was wet sieved with acetone through 100- and 200-mesh sieves (Fisher). The 100—200-mesh fraction was collected and thoroughly washed with acetone to exclude particles smaller than 200 mesh.

All water was distilled in glass following mixed bed deionization. Disposable glass transfer pipets (9 in.) (Rochester Scientific, Rochester, NY, U.S.A.) and glass wool (Corning Glass Works, Corning, NY, U.S.A.) were used as received. All other chemicals were reagent grade and used without further purification. The plasma used (Community Blood Center, Kansas City, MO, U.S.A.) was "recovered human plasma" containing citrate—phosphate—dextrose anticoagulant and was stored at 4°C.

Separation of DHAD from plasma

Disposable columns were prepared by slurry packing 9-in. disposable pipets (containing a small plug of glass wool in the tip) with 150 mg of 100—200-mesh XAD-2 beads suspended in 3—4 ml of methanol. After the bed settled, a second glass wool plug was introduced at the top of the bed to maintain its integrity upon application of aqueous samples. Columns were then sequentially washed with 4—5 column volumes of methanol, distilled water, and 0.05 *M* phosphate buffer (pH 7.4), and stored in this buffer until used.

Freshly prepared plasma samples containing DHAD were stabilized [adjusted to pH 5.3 and made 0.5% (w/v) in ascorbic acid] by the immediate addition of 500 μl of a 5% ascorbic acid solution [prepared in 0.1 *M* citrate buffer (pH 3.0)] to 5-ml plasma samples immediately after the samples were withdrawn. Duplicate 2-ml portions of these samples were then applied directly to the XAD-2 columns and washed with 5-ml portions each of 0.05 *M* ammonium dihydrogen

phosphate (pH 2.7) solution. DHAD was subsequently eluted from the column with 1.75 ml of 2-propanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) (30:70) into a 2-ml volumetric flask and adjusted to volume with the elution solvent.

Chromatography

The eluate from the XAD-2 column containing DHAD was chromatographed as octanesulfonate ion pairs on an octadecylsilane bonded phase column. The chromatographic system was optimized by systematically varying temperature, hetaeron and methanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) composition. The optimum isocratic system for the resolution and quantitation of DHAD in plasma samples was found to consist of methanol—buffer (45:55) containing 6 m*M* sodium octanesulfonate. The flow-rate was maintained at 2.0 ml/min and the column was thermostatted by immersion in a water bath maintained at 49 ± 1°C. All analyses were performed with 100-μl injection volumes.

Quantitative analysis

The concentration of DHAD in a sample was quantitated by comparison of the mean peak area of triplicate injections (100 μl) of the column eluent with a calibration curve prepared in the same manner using samples containing known concentrations of DHAD in plasma. The calibration curve was prepared by the analysis of duplicate samples of DHAD in plasma at each of eight concentrations ranging from 75 to 3000 ng/ml. Samples were prepared by the addition of 0.375—15 μg of DHAD dissolved in 500 μl of 0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7) to sufficient plasma to give a final volume of 5 ml. Ascorbic acid solution (500 μl) was then added and 2-ml aliquots of the final solution were carried through the analysis sequence described above. A calibration curve of DHAD in mobile phase was prepared simultaneously by spiking mobile phase with DHAD (as described for the plasma standard curve) and assaying directly by HPLC. Peak areas were calculated directly with the CDS-111C and measured with a polar planimeter to correlate electronic integration units with peak areas in mm^2.

Stability studies

The stability of DHAD in plasma and plasma adjusted to pH 5.3 [containing 0.5% (w/v) ascorbic acid] at a concentration of 1000 ng/ml was investigated at ambient (ca. 25°C), refrigerator (4°C) and freezer (−17°C) temperatures. Samples stored at these temperatures were assayed for DHAD content at regular time intervals.

RESULTS AND DISCUSSION

The plasma analysis of DHAD consists of four distinct, yet interrelated phases: (a) stabilization of the drug in the sample; (b) separation of drug from the biological matrix; (c) high-efficiency chromatographic separation of drug from potential contaminants; and (d) detection of the drug in the column

effluent with sufficient sensitivity to monitor patient plasma levels after therapeutic dosing at 12 mg/m^2 (ca. 330 μg/kg body weight).

Separation of DHAD from plasma or aqueous buffer

Initial attempts to remove DHAD from aqueous buffer solutions (pH 7.4) by direct extraction met with limited success. Extraction efficiency was better using proton-donating solvents (e.g., chloroform, dichloromethane) than proton-accepting solvents (e.g., diethyl ether, ethyl acetate) and could be further improved by the addition of a second proton-donating species (1-pentanol) to the extractant. However, such extractions were very nonspecific and incomplete, and furthermore, DHAD was shown to have limited stability in plasma (at pH 7.4). DHAD could be quantitatively extracted from pH 4.0 buffer into 1-pentanol as a heptafluorobutyrate ion aggregate. (The stoichiometry of this ion aggregate has not yet been elucidated.) Extending this procedure to plasma, however, necessitated the introduction of a back-extraction of the ion aggregate into 0.05 *M* sulphuric acid to minimize interferences due to coextraction. As a result, in plasma, the overall recovery dropped to 72% and the method was found to be relatively inaccurate (± 11%) and imprecise (coefficient of variance ± 8%). These values are in agreement with the results of Ostroy and Gams [7] for chloroform extraction of DHAD from basic plasma after protein denaturation. Recoveries of 60—80% were reported, and the assay was determined to be reproducible within 10% [7]. In the present study, ion-pair extraction was judged impractical for routine clinical analysis because (1) of the incomplete recovery of DHAD from plasma, (2) the high polarity requirement of the extraction solvent results in non-selective removal of drug with co-extraction of plasma-derived substances, (3) the extent of sample handling leads to long analysis times and increased probability for errors and (4) the noxious odor of 1-pentanol necessitates extractions be carried out in a fume hood (an inconvenience in clinical settings). Therefore, other methods for the removal of DHAD from plasma were sought.

An alternative approach to separation of DHAD from plasma which proved more acceptable involved adsorption of the drug onto beads of the non-ionic resin, Amberlite XAD-2, packed in a short disposable column. The commercially-distributed material is only available in a 20—50-mesh size bead and 500 mg of the dry resin packed in a short cartridge failed to retain a 2-μg load of DHAD. However, reduction of particle size to 100—200 mesh provided an effective column packing for retention of DHAD present in plasma. A disposable column packed with 150 mg of small-particle XAD-2 resin completely retained up to 6 μg of DHAD present in a 2-ml plasma sample.

Plasma samples, containing DHAD, were adjusted to pH 5.3 ± 0.2 and made 0.5% (w/v) in ascorbate (to improve drug stability) and 2 ml of sample was gravity-fed through the XAD-2 column. DHAD was retained by the resin under these conditions and subsequently was eluted with a mixed organic solvent. To minimize co-elution of substances that may pose potential interferences to subsequent steps in the analysis sequence, the column was exhaustively washed with buffer prior to elution of drug. A solvent with minimum elution strength to displace 2 μg of DHAD from 150 mg of XAD-2 resin was systematically sought to further minimize contamination. An acidic eluent [0.05 *M* am-

monium dihydrogen phosphate (pH 2.7)] was chosen to present the amine functions in an ionized state, which would result both in minimizing the attraction of the drug for the hydrophobic surface of the resin and in stabilizing the drug against pH-dependent oxidative degradation. Elution strength was increased by the addition of 2-propanol to the buffer. Complete elution and recovery of the drug was achieved with minimum contamination of the sample using 1.75 ml of eluent at a composition of 30% 2-propanol in 0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7). Higher concentrations of 2-propanol were less suitable since they caused co-elution of contaminants from the resin and adversely affected the shape and resolution of the HPLC bands due to the resulting increase in the strength of the injection solvent.

Chromatography

Separation of DHAD from plasma contaminants, chemical impurities and degradation products, which co-eluted from the XAD-2 columns, was achieved by isocratic reversed-phase paired-ion chromatography on an RP-18 column.

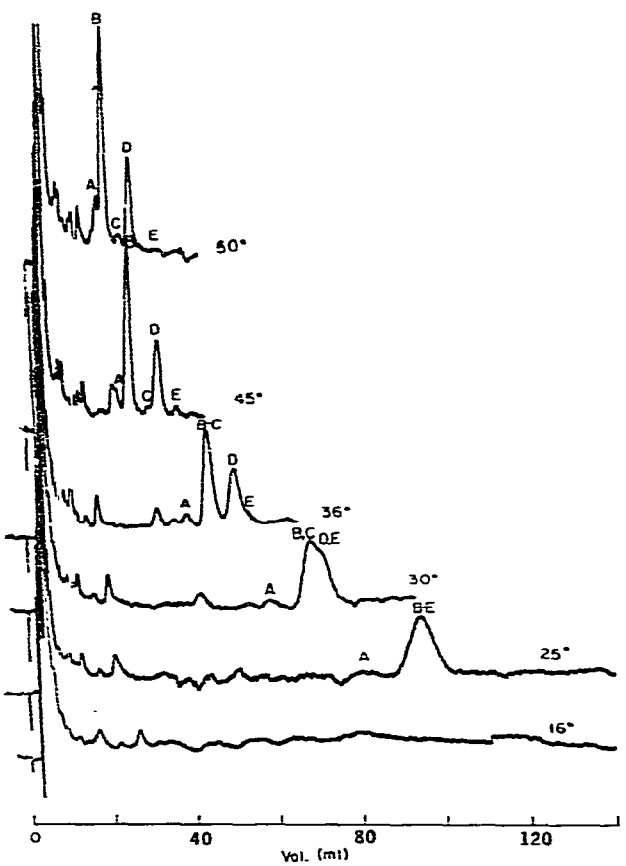


Fig. 1. Chromatograms of DHAD species obtained from a plasma sample carried through the analysis sequence using a mobile phase of 6 m*M* sodium octanesulfonate in methanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) (45 : 55). A and C are impurities, B is DHAD and D and E are chemical degradation products of the parent drug.

Temperature effects

When chromatography was carried out at ambient temperature (25 ±1°C), resolution was unsatisfactory using a variety of stationary phases (μ-CN, RP-18, RP-8), mobile phases (containing methanol, tetrahydrofuran, 2-propanol or acetonitrile as organic modifier), buffers of differing pH and ion-pairing agents (C_4—C_8 sulfonates). Day-to-day variations in room temperature appeared to have an effect on the quality of separation, prompting an investigation of temperature effects on chromatographic selectivity of the paired-ion system. An RP-18 column and a mobile phase of methanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) (45:55) made 6 m*M* in sodium octanesulfonate was used to isolate DHAD from a plasma sample that had been previously carried through XAD-2 column clean-up. An apparent single peak (B—E), preceded by a shoulder (A) (Fig. 1) was observed at ambient temperature (25°C). As shown in Figs. 1 and 2, an increase in temperature resulted in an improvement in resolution as evidenced by the emergence of peaks C, D and E from under the DHAD peak (B).

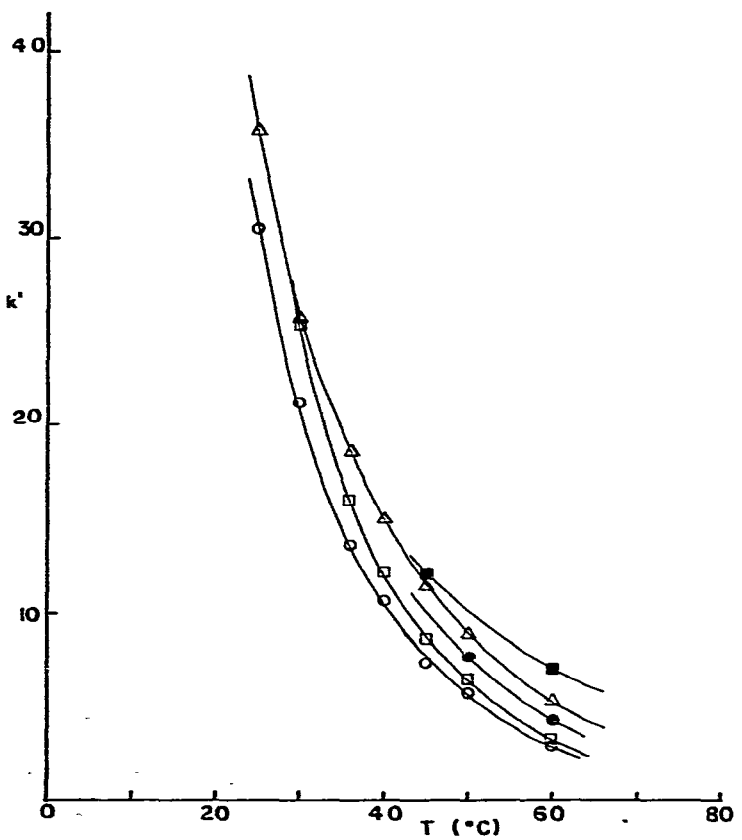


Fig. 2. Capacity factor (*k'*) for DHAD species vs. temperature for separation of DHAD (□), impurities A (○) and C (●), and degradation products D (△) and E (■) on an RP-18 column using methanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) (45:55) containing 6 m*M* sodium octanesulfonate as mobile phase.

The dependency of capacity factor (k') on temperature is given by

$$k'_A = \Phi \exp(-\Delta H^0_A/RT) \exp(\Delta S^0_A/R) \quad (1)$$

where Φ is the phase ratio of the column and ΔH^0_A and ΔS^0_A are the enthalpy and entropy of transfer of solute A between chromatographic phases, respectively. In the present case, as for the partitioning of other ion aggregates [8], the distribution process appears to be enthalpically-controlled. The observed decrease in k' with increasing temperature (Figs. 1 and 2) indicates that the partitioning process for all five analytes (A—E) is exothermic. The magnitude of the enthalpy of transfer for the individual components is different as determined from Van 't Hoff plots (ln k' vs. the reciprocal of the absolute temperature) prepared from data for compounds A—E (Fig. 3). Thus temperature changes would be expected to affect k' for each analyte differently, providing a means for modifying selectivity, as exhibited in Figs. 1 and 2. Although similar temperature effects have been observed in reversed-phase chromatography [6,9—11] such behavior has not been described for reversed-phase paired-ion HPLC.

Temperature elevation also decreases the viscosity of the mobile phase and minimizes mass transfer effects in both stationary and mobile phases [12]. These effects are reflected in an increased plate count (N; Fig. 4) being observed with increased temperature. The dramatic increase is, in part, artifactual since it also represents the resolution of two or more superimposed bands. The decrease in chromatographic efficiency observed above 50°C can probably be attributed to (a) a loss in the effective number of theoretical plates (N_{eff}) caused by the decrease in k' with increasing temperature as described by eqn. 2 [13] and (b) extracolumn effects which become pronounced at $k' \leqslant 2$ [13]

$$N_{eff} = N\left(\frac{k'}{1+k'}\right)^2 \quad (2)$$

Temperature increases resulted in an increase in peak skewness, defined by the asymmetry factors of 1.2 at 25°C, 1.7 at 50°C, and 2.1 at 60°C. Thus, the change in peak shape that occurs with changes in temperature does not account for the loss in apparent plate count at higher temperatures. Optimum resolution and chromatographic efficiency was achieved at a temperature of 49 ± 1°C which was used for subsequent development of chromatographic separations.

Choice of ion-pairing agent

The k' values for the three DHAD-derived species and the two impurities increased with increasing length of the carbon chain of the alkylsulfonate hetaeron (Fig. 5 a—d) and with hetaeron concentration (Fig. 6). Increasing the chain length of the counter ion increased k' by increasing the activity coefficient of the hetaeron in the mobile phase, thus favoring the partitioning of the ion pair into the stationary phase. Neither butane-, pentane- nor hexanesulfonate (Fig. 5 a—c) offered resolution of the five species (A—E) within an acceptable range of k' values ($1 < k' < 10$) due to the large difference in partitioning behavior for peaks A—C and D—E. As the carbon chain length of the hetaeron was increased, k' for all five components could be made more similar

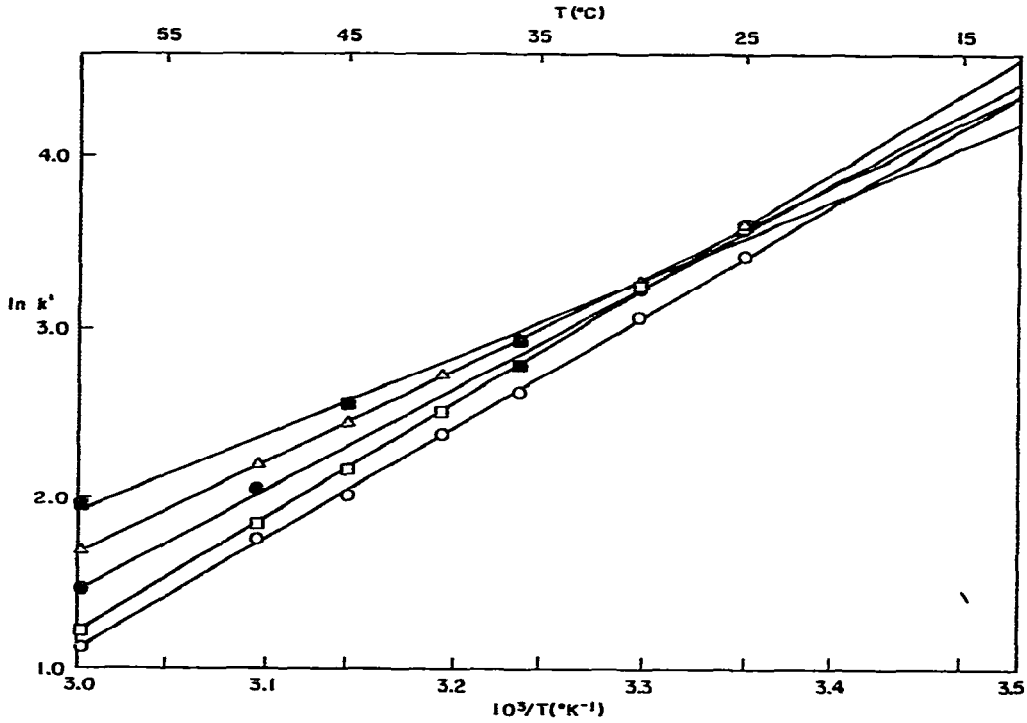


Fig. 3. Van 't Hoff plot for DHAD (□); impurities A (○) and C (●); and degradation products D (△) and E (■). Mobile phase: methanol—0.05 *M* $NH_4H_2PO_4$ (pH 2.70) (45:55) + 6 m*M* sodium octanesulfonate; flow-rate: 2.0 ml/min.

(while still maintaining peak resolution) by carrying out the separation at a higher concentration of organic modifier. Octanesulfonic acid provided acceptable resolution of all desired components (Fig. 5d).

The capacity factor of the ion pair is related to its partition coefficient, K_p, according to eqn. 3 [13]

$$k' = \frac{V_s}{V_m} K_p[B^-]^x_{mp} \qquad (3)$$

where V_s and V_m are the volumes of the stationary and mobile phases, respectively. Thus, k' should be proportional to the concentration of the hetaeron, $[B^-]$, in the mobile phase. Plots of log k' vs. log $[B^-]_{mp}$ were linear with slopes close to 1, indicating the stoichiometry of DHAD to hetaeron in the ion aggregate was 1:1. As the octanesulfonate concentration was increased from 3 to 9 m*M* (Fig. 6), the k' value for DHAD increased at equivalent methanol concentrations. However, at higher hetaeron concentrations, resolution of neighboring bands decreased, apparently due to increasing similarity in the extent of ion-pairing of the different species. As the concentration of hetaeron increased, k' for DHAD increased faster than k' for the major degradation peak (D), thus providing an additional source of separation selectivity.

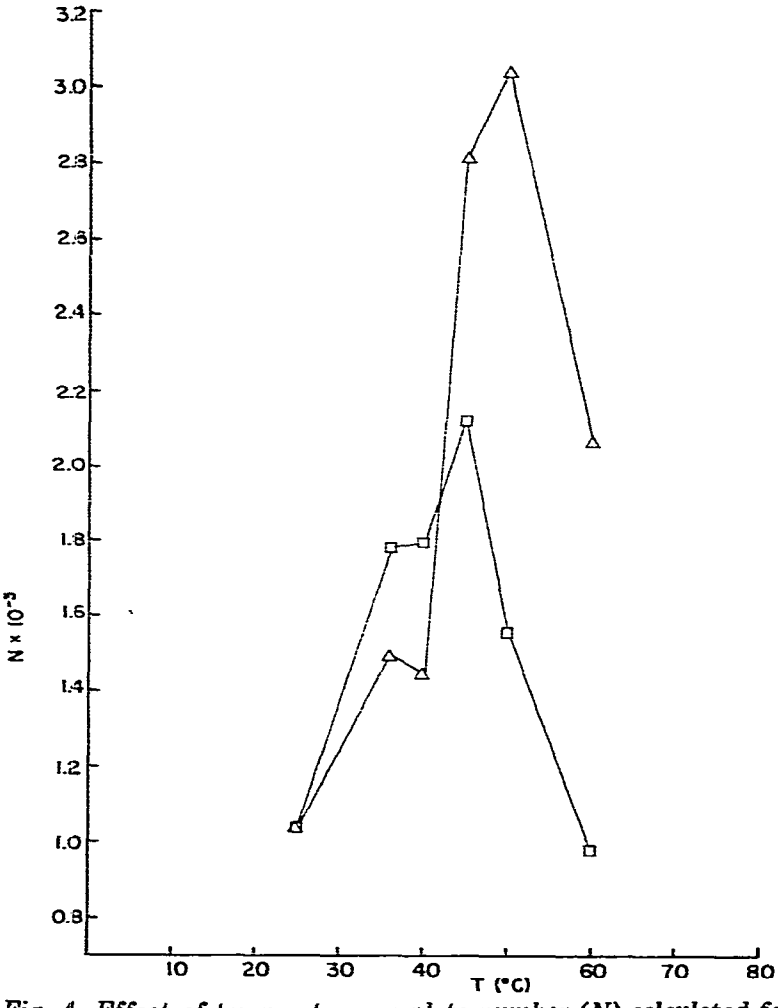


Fig. 4. Effect of temperature on plate number (N) calculated for DHAD (□) and its major degradation product, D (△).

Chromatographic results

Maximum resolution of components coupled with minimum analysis time was achieved using a mobile phase of methanol—0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7) (45:55) made 6 m*M* in sodium octanesulfonate. A chromatogram of a plasma sample containing 500 ng of DHAD per ml that had been carried through the analysis sequence is shown in Fig. 7 and contrasted with a plasma blank, carried through the analysis scheme but containing no drug. No contamination is seen in the region in which DHAD elutes. Under the chromatographic conditions stated, DHAD elutes with a capacity factor (k') of 6.4 (V_R = 16.8 ml). The analytical column was calculated to possess ca. 2125 plates (N) (reduced plate height, h = 14.1). However, the peak asymmetry factor for the DHAD peak is ca. 1.7 suggesting that a significant positive error in plate count may have been produced in calculation due to use of the simplified expression, $N = 16\ (t_r/t_w)^2$ [13]. Peaks A ($k' = 5.8$) and C ($k' = 7.7$) are apparent

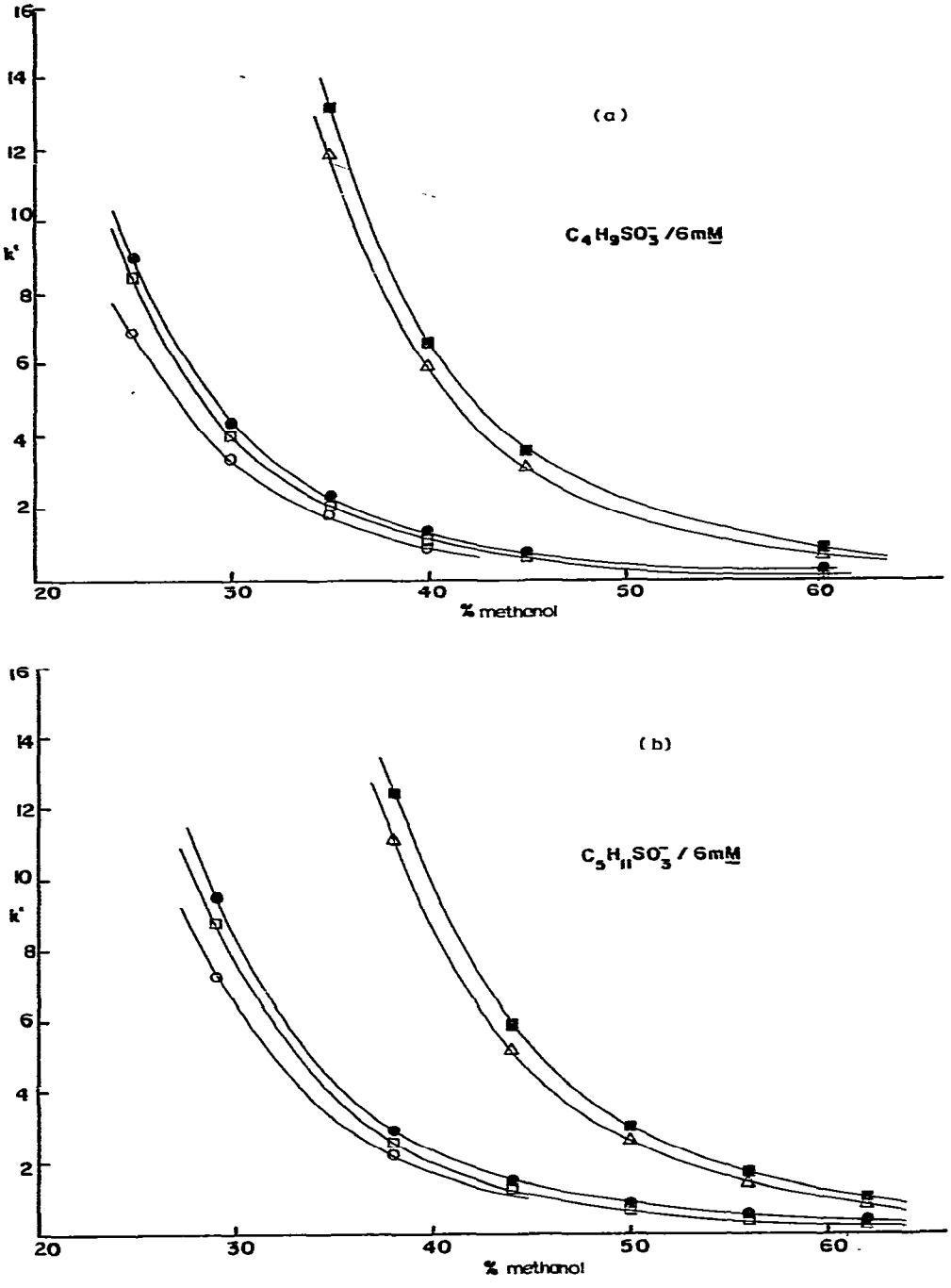
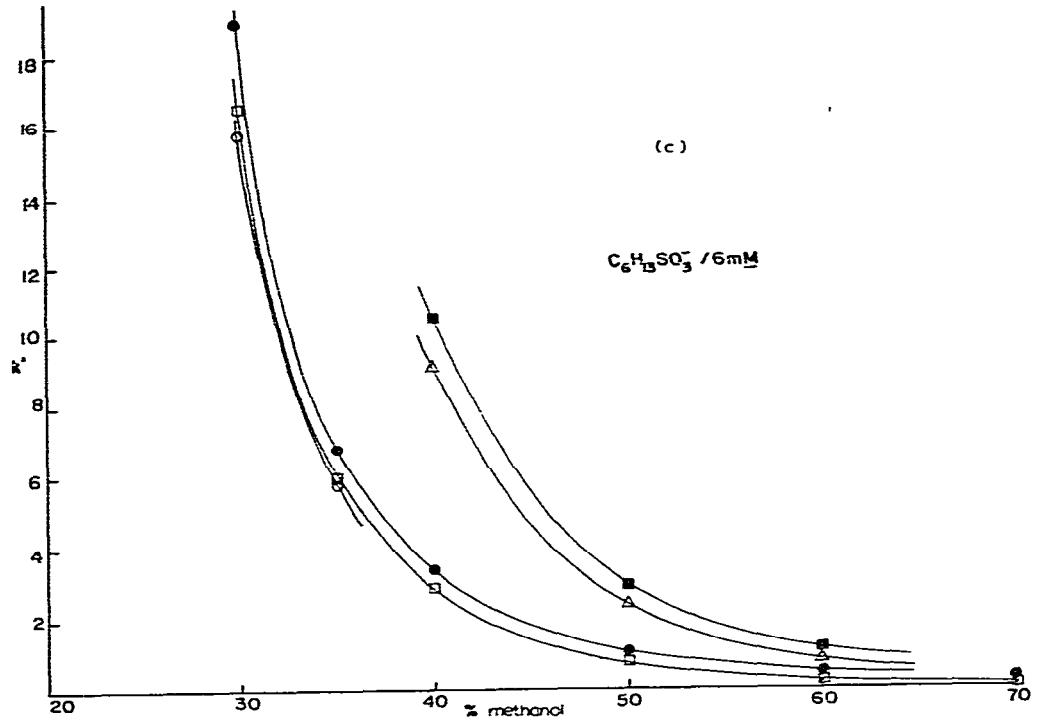


Fig. 5. Capacity factor (k') for impurities A (○) and C (●), DHAD (□), and its degradation products D (△) and E (■) vs. methanol concentration in mobile phase of 0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7) and 6 m*M* sodium alkanesulfonate. (a) Sodium butanesulfonate; (b) sodium pentanesulfonate; (c) sodium hexanesulfonate; (d) sodium octanesulfonate. Column thermostatted at 49 ± 1°C.



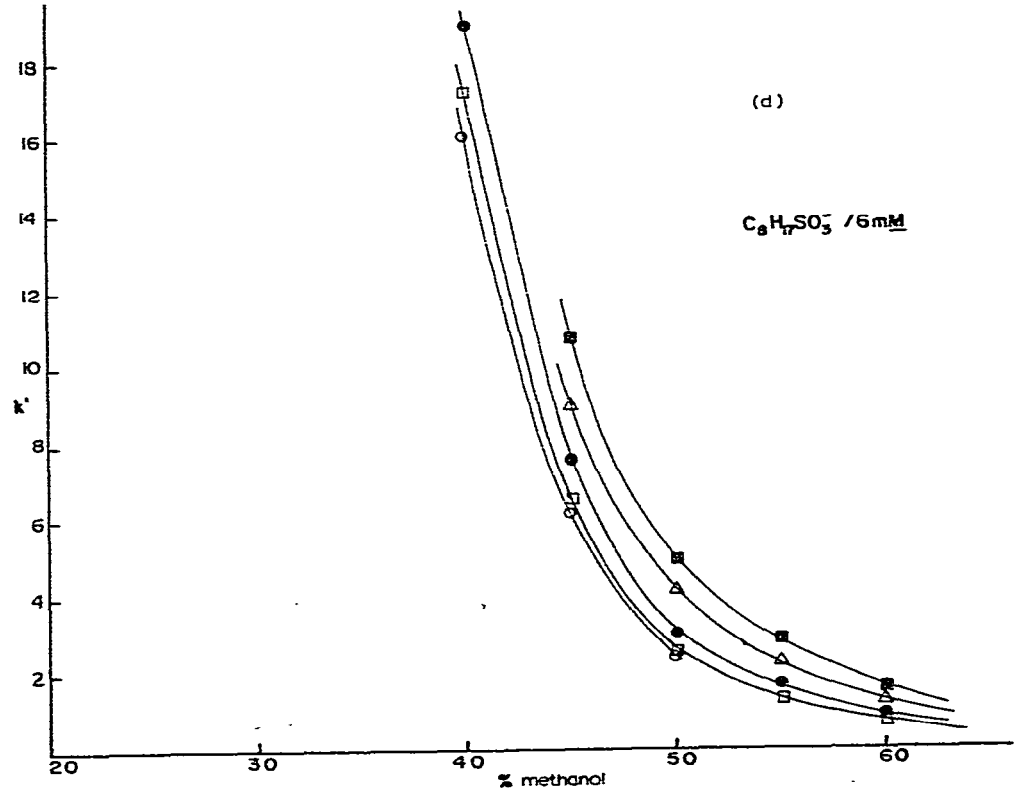


Fig. 5 (continued).

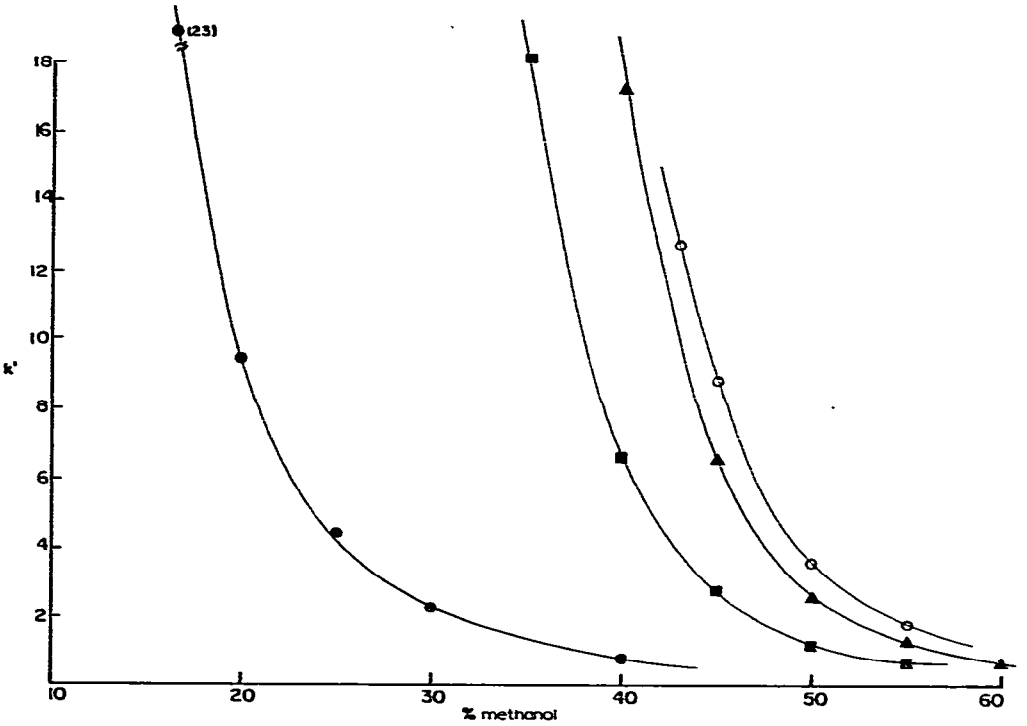


Fig. 6. Capacity factor (k') for DHAD vs. methanol concentration in mobile phase of 0.05 *M* ammonium dihydrogen phosphate buffer (pH 2.7) containing sodium octanesulfonate at concentrations of 0 (•), 3 (▪), 6 (▲) and 9 (○) m*M*. System thermostatted at 49 ± 1°C.

impurities in the drug sample. They are present immediately after the drug is dissolved in buffer or plasma and do not appear to change in intensity as a function of time. Components D ($k' = 8.9$) and E ($k' = 10.2$) were not initially present and their concentrations increase with time, concomitant with a decrease in the amount of DHAD present. Consequently, it appears that they are products of chemical degradation of DHAD.

Quantitative analysis

DHAD was quantitated in plasma by comparison of computer-calculated peak areas for the analyte to a standard curve constructed from the analysis of plasma samples containing known amounts of drug. The area of the DHAD peak was linearly related to DHAD concentration for 8 concentrations of drug in the range 75—3000 ng of DHAD per ml of plasma. Linearity of response was determined by least squares analysis of data points, and is described by the line $y = 4347\,x - 103890$ (correlation coefficient > 0.999), with the line crossing the abscissa at 24 ng/ml. A similar curve was prepared for DHAD in the HPLC mobile phase, yielding a line described by $y = 4583\,x - 11198$ (correlation coefficient > 0.999), with the line crossing the abscissa at 2.4 ng/ml. Absolute recovery of DHAD from plasma was evaluated by comparing the slopes of these two lines, and indicated an overall recovery of ca. 95%. Peak areas in mm^2 were measured via polar planimetry at a chart speed of 0.2 in./min and yielded lines

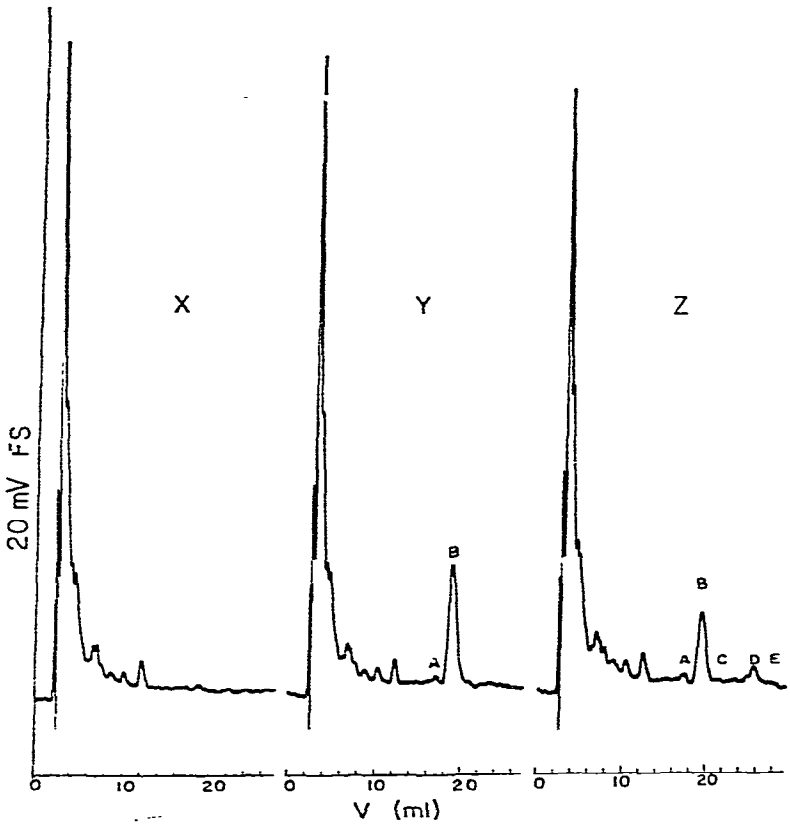


Fig. 7. Chromatogram of DHAD from plasma. Tracing X represents a blank, i.e. biological fluid not containing drug but carried through the analysis sequence; tracing Y is the result obtained from work-up of a fresh plasma sample containing 500 ng of DHAD per ml (50 ng drug placed on column). Tracing Z differs from Y only in that the sample had aged for 3 h at 37°C before work-up. Peaks A and C are impurities in the drug; B is DHAD and D and E are chemical degradation products of the parent drug. Separation was carried out on an RP-18 bonded phase column with 6 m*M* sodium octanesulfonate in methanol—ammonium dihydrogen phosphate buffer (0.05 *M*; pH 2.7) (45:55) as mobile phase. Flow-rate: 2 ml/min. System thermostatted at 49 ± 1°C.

of $y = 0.301\,x - 7.186$ and $y = 0.317\,x - 0.751$ for the curves from plasma and mobile phase, respectively. Detection limits were approximately 75 ng/ml plasma (ca. 7.5 ng drug applied to the column in 100-µl injection volume) at the 3σ level, as determined by the analysis of plasma samples supplemented with drug at these levels. Plasma samples at the 75 ng/ml level could be analyzed with a precision of ± 10%, whereas samples at 3000 ng/ml could be analyzed with ± 2% precision.

Stability studies

At room temperature (25°C), DHAD was found to be unstable in aqueous solution, degrading in an apparent first-order manner with $t_{\frac{1}{2}} \approx 130$ h in 0.05 *M* phosphate buffer (pH 7.4) and $t_{\frac{1}{2}} \approx 24$ h under similar conditions in

plasma. The loss of drug could be reduced by storing samples at lower temperatures. When refrigerated (4°C), the $t_{\frac{1}{2}}$ of DHAD was extended to 6 days (60% loss in one week) and at freezer temperature (—17°C), 13% of drug was lost in one week ($t_{\frac{1}{2}} \approx 36$ days). This instability makes proper storage conditions imparative to prevent drug loss from the sample prior to analysis. Degradation probably involves oxidation of the phenylenediamine moiety to the corresponding quinoneimine which is subject to hydrolysis to yield the quinone [14]. Since such reactions are normally pH-dependent [15], it was felt that stability could be enhanced by decreasing pH. In plasma adjusted to pH 5.3, the $t_{\frac{1}{2}}$ for loss of DHAD at room temperature increased by a factor of 2.5 (to ca. 60 h) relative to that in plasma at pH 7.4. Further reduction of pH was not practical since at pH < 5.3, precipitation of the biological material occurred, seriously complicating homogenous sampling of the plasma.

Since degradation was presumed to involve oxidation, the use of antioxidants to further stabilize DHAD in plasma samples was studied. The first candidate, sodium bisulfite (2.6%, w/v) rapidly reacted ($t_{\frac{1}{2}} \approx 20$ min) with DHAD in phosphate buffer (pH 7.4) and was, therefore, unsuitable. Ascorbic acid proved to be a much more useful reagent, but due to its instability at neutral pH [16], acidification of plasma samples concurrent with the addition of ascorbate was necessary. In plasma adjusted to pH 5.3, the addition of 0.5% (w/v) ascorbate stabilized the system and resulted in less than 1% loss of DHAD in 48 h at room temperature. At refrigerator temperatures (ca. 4°C), less than 4% loss of DHAD was observed in one week in these same plasma samples. To determine whether or not ascorbate could reduce the oxidized product of DHAD back to parent drug, thereby introducing potential positive deviations in analysis, ascorbate was added to a plasma sample (pH 5.3) containing DHAD which had been allowed to degrade to 50% of its initial concentration. Over a 24-h period at room temperature, ascorbate failed to regenerate significant quantities of DHAD, but did prevent its further degradation thus demonstrating its effectiveness as a stabilizer for DHAD in plasma. In view of these results, it is clear that in all clinical studies, freshly obtained plasma samples should be immediately stabilized through pH adjustment and addition of ascorbic acid.

In summary, a rapid clinical method is described for monitoring the anticancer agent DHAD in plasma. The drug was separated from plasma constituents by its retention on a disposable glass column packed with XAD-2 beads. Subsequent elution followed by isothermal and isocratic HPLC analysis of the eluent from the XAD-2 column (with spectrophotometric detection) provided a sensitive and specific means for measuring DHAD at levels $\geqslant 75$ ng/ml in plasma with a precision of $\pm 10\%$. Although DHAD is unstable in biological media, a procedure for stabilization involving addition of ascorbic acid together with acidification provides adequate sample stability. In a previous report [7] of a clinical method for DHAD, the drug stability in plasma was not addressed and results obtained may be in question. Overall, the method presented in this paper appears to offer significant advantages over the works presented thus far in the literature [6,7] and may, therefore, be more applicable for the routine clinical analysis of DHAD.

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