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AMMONIUM BICARBONATE MEDIATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY RESOLUTION OF BIS-ANTHRACYCLINES

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SUMMARY

Bis-anthracyclines derived from the anti-cancer drug daunomycin have been resolved from reactants and intermediates using low levels of ammonium bicarbonate as a volatile pseudo ion-pairing agent under reversed-phase C_{18} high-performance liquid chromatographic conditions. The resolution and selectivity of this system is enhanced by increasing the number of polar groups on the stationary phase, and by decreasing the bicarbonate concentration. These conditions enable optimal selectivity to be established to achieve quantitative semi-preparative recovery of bis-anthracycline drugs, with minimal degradation during solvent removal. This system offers potential for the resolution and isolation of a number of unstable anthracycline and bis-anthracycline drugs.

INTRODUCTION

The anthracyclines [daunomycin (I) and adriamycin (II), Fig. 1] are important anti-cancer agents and are the most widely used DNA intercalating agents in current clinical use[1–4]. Because of associated side effects, particularly cardiotoxicity[5–8], there has been an enormous effort to find derivatives of these drugs which are more effective or less cardiotoxic. For this reason, we have synthesized a range of peptidic bis-daunomycin derivatives (Fig. 1) using the method outlined previously for methylene linked bis-anthracyclines[9,10]. These derivatives are bis-intercalators which exhibit an extremely high affinity for DNA (10^9 to 10^{10} M^{-1}), dissociating some 1500 times slower from DNA than the parent anthracycline[10,11].

The bis-anthracyclines were initially purified by semi-preparative high-performance liquid chromatography (HPLC) with a reversed-phase C_{18} column and isocratic methanol–water (60:40)–ammonium carbonate solvent system previously used in our laboratories for methylene linked bis-anthracyclines[9]. This system adequately resolved the reactant (I) and the peptidic mono- (III) and bis-anthracycline (IV) products. When drying semi-preparative HPLC fractions containing a bis-anthracycline (IV), however, it was found that as the solvent was removed the product (IV) decomposed to (I) and (III) as the percentage of ammonium carbonate increased.

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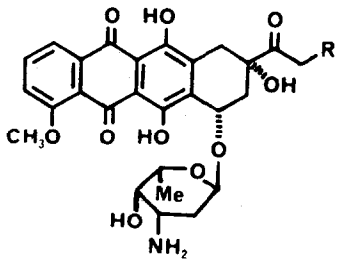
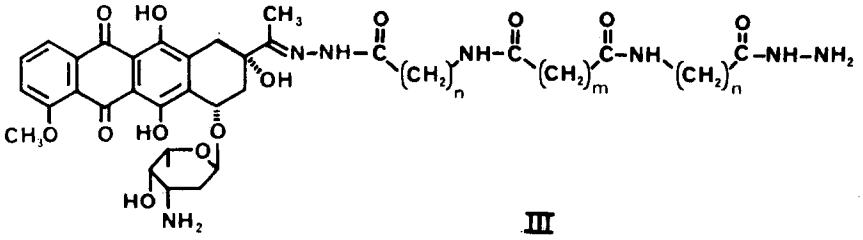
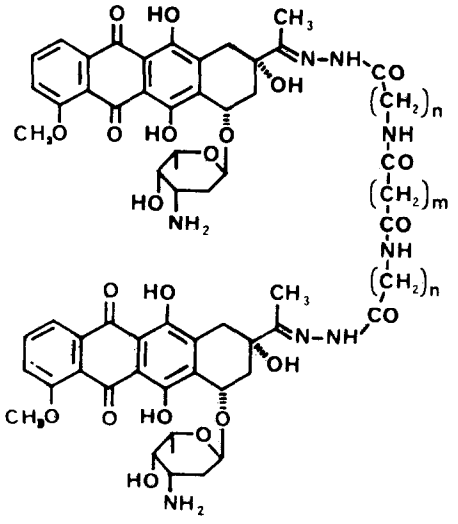


I, R = H

II, R = OH



III



IV

Fig. 1. Structures of the anthracyclines daunomycin (I), adriamycin (II), the peptidic mono- (III) and bis- (IV) daunomycin derivatives.

In addition, the overall time taken and the temperature at which the process was carried out at were found to influence the extent of decomposition. This degradation was attributed to the instability of the hydrazone under the extremes of pH which

accompanies the increase in the concentration of the ammonium carbonate, present in the mobile phase, prior to decomposition of the buffer itself.

We therefore sought a method based on our earlier criteria[9] for handling bis-anthracyclines (*i.e.* baseline resolution of all reactants and products; rapid, simple and inexpensive; volatile solvent buffer system) with the additional requirements that the mobile phase be less degradative to the column (*i.e.* increase column life-time) and to the bis-anthracyclines in the final drying step.

To this end, ammonium bicarbonate was chosen as an alternative buffer since it is more stable to air than ammonium carbonate and enables solutions of a reproducible pH to be prepared[12]. More importantly the pH of these solutions is lower than comparable ones prepared from ammonium carbonate. The use of ammonium bicarbonate results in both minimal and reproducable base induced degradation during solvent removal and offers improved resolution in comparison to that obtained using an equivalent amount of the carbonate buffer. Because of these advantages the bicarbonate buffer was used for all further studies.

We wished to fully investigate the effect of bicarbonate levels on resolving individual components of a reaction mixture in conjunction with evaluating two C_{18} analytical columns with differing characteristics. From this systematic study, optimal conditions for the recovery of bis-anthracyclines of this type were established and transferred without further modification to a semi-preparative column.

EXPERIMENTAL

Materials

Daunomycin was supplied by Farmitalia-Carlo Erba (Milan, Italy) and was used without further purification. Analytical reagent grade ammonium bicarbonate (99%) and ammonium acetate (98%) were obtained from BDH and M&B respectively and used without further purification. HPLC-grade solvents were obtained from Waters Assoc. The water used in the HPLC solvent systems was obtained from a 4-bowl Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.) fitted with a charcoal, ion-exchange (2) and Organex-Q cartridges and a 0.2-μm filter.

Equipment

The HPLC system used consisted of a U6K injector, an M-45 pump and a Model 450 variable-wavelength detector, all from Waters Assoc. The columns used were a 10-μm C_{18} Z-Pak cartridge in a Z-module system, a 4-μm, 30 cm × 3.9 mm I.D. Nova-Pak C_{18} steel column (exhaustively end-capped) and a 30 cm × 7.8 mm I.D. μBondapak C_{18} steel semi-preparative column (10 μm), all from Waters Assoc. All analyses were carried out in an air-conditioned room at 20°C.

Methods

All samples were taken from one reaction mixture ($n = 1$, $m = 2$ in Fig. 1) and stored at −20°C when not in use. Injection volumes were 10 μl for the analytical columns and 1 ml for the semi-preparative column. Flow-rates were 0.5 ml/min and 2.0 ml/min for the Nova-Pak and Z-Pak analytical columns respectively, and 2.0 ml/min for the semi-preparative columns. Buffers were prepared daily with Milli-Q water, then mixed with the organic solvent and degassed immediately before use. The

mobile phase was methanol–water (60:40, v/v), with varying amounts of ammonium bicarbonate as noted in the text. Resolution (R) was calculated according to the following relationship[13]:

$$R = \frac{\Delta t}{\frac{w_{1,1/2} + w_{2,1/2}}{2}}$$

where Δt is the difference in retention times (cm of chart between two peaks) and w the half height peak width (cm) of peaks 1 and 2.

Samples isolated by semi-preparative HPLC were collected in a precooled flask (−77°C, acetone–carbon dioxide). The solvent was removed *in vacuo* (10^{-3} mmHg) with rapid stirring while maintaining the temperature of the vessel between 10–15°C. The ammonium bicarbonate was decomposed by immersing the flask (still under vacuum) in a water bath at 60°C for 10–15 min. The residue was dissolved in milli-Q water and the pH adjusted to 6.8 using 0.1 M hydrochloric acid. The solution was then concentrated under the above conditions and the purity of the sample determined by analytical HPLC using a C_{18} Z-Pak column and eluent consisting of methanol–water (60:40), containing 3% ammonium acetate.

RESULTS AND DISCUSSION

Capacity ratio

For all columns employed, three major peaks were detected, corresponding to the mono-anthracycline (III) (linker with only one daunomycin appended to one end), bis-daunomycin (IV) and unreacted daunomycin (I) (Fig. 2). These assignments

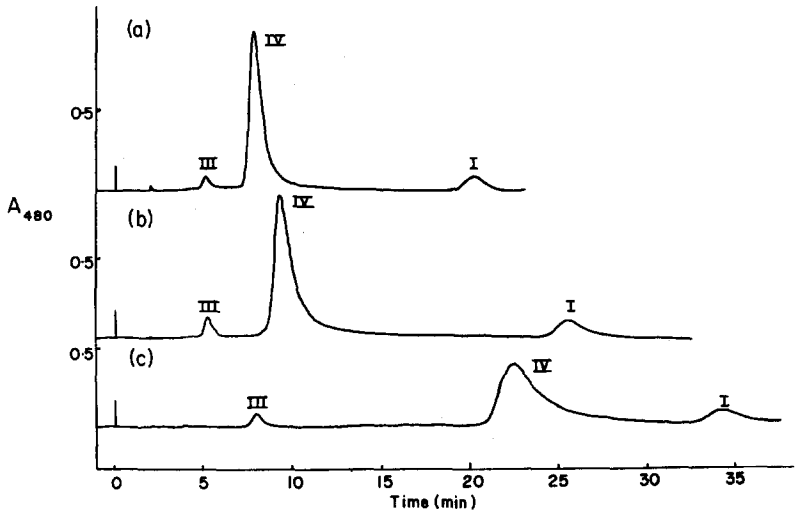


Fig. 2. Analytical HPLC traces of the mixture of anthracyclines (I), (III) and (IV) (n = 1, m = 2 in Fig. 1) on a Waters Z-Pak C_{18} column eluted at 2.0 ml/min with methanol–water (60:40) containing (a) 1.0%, (b) 0.1% and (c) 0.05% ammonium bicarbonate buffer.

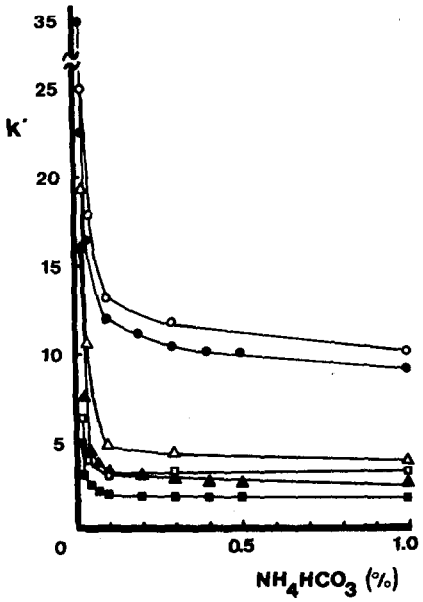


Fig. 3. Capacity ratios (k') of the three anthracyclines at varying concentrations of ammonium bicarbonate for the Z-Pak [○, (I); □, (III); △, (IV)] and Nova-Pak [●, (I); ■, (III); ▲, (IV)] C_{18} columns.

were based on ^{13}C NMR, ^{1}H NMR and fast atom bombardment mass spectra of each component[10]. The capacity ratios (k') of these peaks are shown in Fig. 3 for both analytical columns used in this work at varying concentrations of ammonium bicarbonate. The retention times of all components increases with the decrease of ammonium bicarbonate, as expected for limiting amounts of ion-pairing and ion-shielding capacity. Below 0.1%, this effect becomes pronounced, and leads to retention times of up to ten times longer than those obtained at the 1% buffer levels, as routinely used previously[9]. Bis-antracyclines are doubly charged compounds and have a variety of polar functional groups in addition to the dominant hydrophobic aromatic ring system. It was therefore expected that use of a Nova-Pak C_{18} column which is more extensively end-capped than the C_{18} μBondapak column, would result in shorter retention times commensurate with a decreased contribution from polar contributions to the separation. This is indeed observed for all three components, at all concentrations of bicarbonate.

Selectivity

Previous work with bis-anthracyclines has shown that mono-anthracycline and bis-anthracyclines can be difficult to resolve (at a semi-preparative level), depending on the nature of the linker in the bis-drug. There is a loss of selectivity (α) with increasing linker length (Fig. 4), and this suggests potential problems for semi-preparative purification for longer chain length compounds. It was therefore a high priority to establish conditions which yielded maximal selectivity for this pair of compounds. The effect of ammonium bicarbonate concentration on the selectivity and resolution of these drugs is shown in Figs. 5 and 6, respectively. At low bicar-

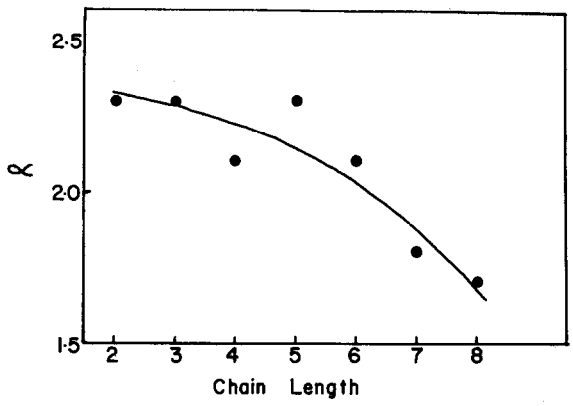
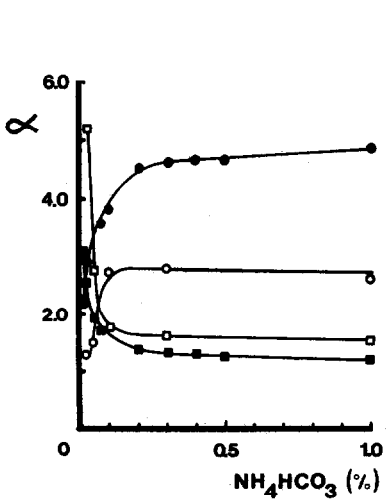


Fig. 4. Dependence of selectivity (α) for (III) and (IV) on chain length (*m* in Fig. 1).

bonate levels in the mobile phase, the selectivity of the mono–bis pair can be chosen at will, since the selectivity varies from 1.8 to greater than 5 for the Z-Pak C_{18} column. The enhanced selectivity is most apparent for the Z-Pak column which exhibits a greater mixed mode of separation (both adsorption and partitioning) compared to the exhaustively end-capped Nova-Pak column.

Resolution

The resolution (Fig. 6) of the mono-daunomycin–bis-daunomycin pair, calculated by the half-height method, shows a small linear improvement with decreasing ammonium bicarbonate for the Nova-Pak C_{18} column. In contrast, the Z-Pak C_{18}



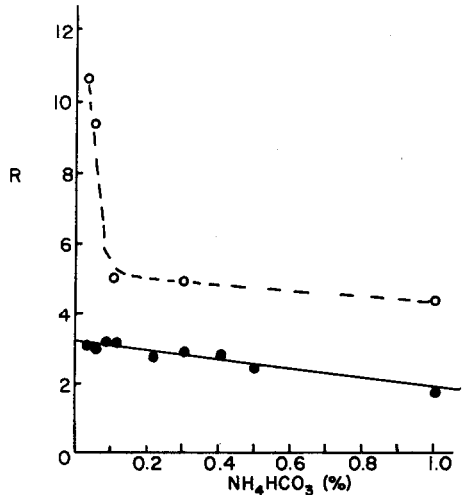


Fig. 5. Variation in selectivity (α) with concentration of ammonium bicarbonate for the Z-Pak [(I)–(IV), ○; (IV)–(III), □] and Nova-Pak [(I)–(IV), ●; (IV)–(III), ■] C_{18} columns.

Fig. 6. Resolution (*R*) of the mono–bis anthracycline pair [(III)–(IV)] at varying concentrations of ammonium bicarbonate for the Z-Pak (○) and Nova-Pak (●) C_{18} columns.

column exhibits superior resolution for the compounds of interest, especially at low ammonium bicarbonate levels (below 0.1%) where there is a profound dependence on buffer concentration.

Yield, purity and degradation

The dependence of selectivity for the mono–bis drug pair on mobile phase conditions offers considerable potential for semi-preparative applications with these drugs. Compounds which were previously difficult to separate can now be readily isolated by decreasing the mobile phase bicarbonate level appropriately. Furthermore, complete recovery of bis-anthracyclines is now possible without losses from overlap with the mono-derivative (this is of particular importance when the mono-compound is the major component and "tails" into the bis-peak). In general, the ability to choose any desired selectivity for this pair of compounds means that column loading can be increased substantially, without suffering any concommitant losses of purity of the bis-anthracycline.

This procedure (*i.e.* low bicarbonate) results in quantitative recovery of bis-anthracyclines and purity typically greater than 97% after solvent removal. These recoveries are substantially better than achieved with earlier semi-preparative work with these drugs.

CONCLUSIONS

Ammonium bicarbonate acts as an ionic modifier by shielding polar or charged groups, and also by its role as a pseudo ion-pair. Since the capacity factor, selectivity and resolution of bis- and mono-anthracycline derivatives are all enhanced at low buffer concentration, this supports the notion of a mixed mode of separation (both adsorption and partitioning). Reversed-phase C_{18} columns therefore offer better characteristics for these polar, aromatic drugs if a significant number of silanol groups are present on the stationary phase.

Maximal contributions from polar groups on both the drugs and stationary phase, occurs at low buffer concentration. For this reason, these drugs are best separated using the lowest concentration of ammonium bicarbonate possible, within the constraints of extreme broadening, tailing and long retention times which accompany such a decrease. As a result of the high selectivity associated with decreasing the level of ammonium bicarbonate it is now possible to obtain good separation of components which were previously unresolvable at higher buffer concentrations. For the same reason, an increased column loading is also possible.

The major advantage of this improved resolution offered by low buffer levels, is the ability to obtain 100% recovery of the bis-anthracycline while minimizing subsequent losses from degradation during drying because of the presence of only small amounts of the buffer. In addition, the less alkaline conditions arising from using only minimal amounts of bicarbonate serve to increase the workable life span of the column.

In summary, extremely low levels of bicarbonate offers four major advantages over the procedure previously used for semi-preparative work with bis-anthracyclines: quantitative recovery, increased column loading, less degradation of the product and an increased column life-time.

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