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APPLICATION OF ANALYTICAL AND SEMI-PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO ANTHRACYCLINES AND BIS-ANTHRACYCLINE DERIVATIVES

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SUMMARY

Efficient high-performance liquid chromatographic (HPLC) methods have been developed for routine analysis of a number of anthracycline derivatives using reversed-phase μBondapak C_{18} columns and an isocratic methanol–water–ammonium carbonate (or acetate) solvent system. The rates of formation of bis-daunomycin derivatives of α,ω-dicarboxylic acid hydrazides have been investigated using the analytical methods developed. It has been demonstrated that the reaction proceeds via an intermediate mono-hydrazone. Both the mono- and bis-hydrazones have been isolated by preparative HPLC.

INTRODUCTION

Daunomycin (daunorubicin) and adriamycin (doxorubicin) are anthracycline drugs (structures shown in Fig. 1) which are effective anti-cancer agents and have been in clinical use for over a decade[1–4]. The chemical[5], physico-chemical[6] and biochemical[6–8] effects of these drugs have recently been reviewed, as has their mode of action[6–9]. Unfortunately, both drugs are cardiotoxic[6–9], and this necessitates restriction of their use to a maximum accumulative dose of 550 mg/m^2 of body surface area[9]. This means that their full potential can not be realised because of this limiting side effect. One of the major strategies developed to overcome this limitation has been the search for more effective or less cardiotoxic derivatives[2,3,5,10]. Another approach used attempts to define the biological nature of the cardiotoxicity in order to allow concurrent administration of appropriate inhibitors[7].

In parallel with the approaches to overcome the cardiotoxicity problem, there has been a great deal of investigation of the pharmacokinetic behaviour of these drugs[11–14]. These studies initially quantitated only the total amount of drug chromophore present. However, more recently, thin-layer chromatography (TLC)[11], and subsequently high-performance liquid chromatography (HPLC), have been utilized to enable separation and quantitation of a range of drug metabolites present in mammalian blood and urine. Early HPLC procedures were aimed at optimizing the reso-

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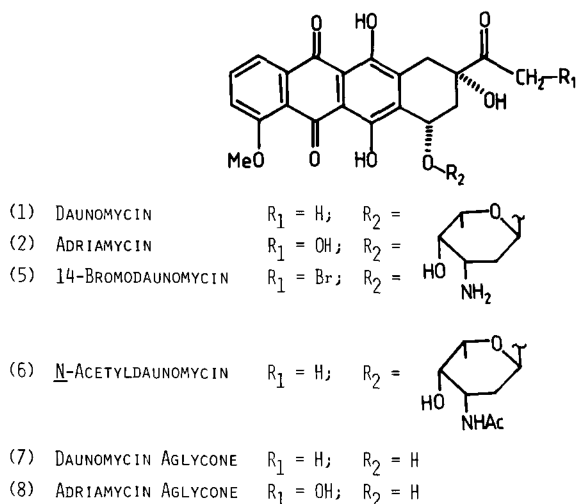

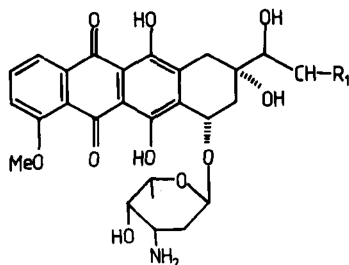

Fig. 1. Structure of anthracyclines and derivatives.

lution of the major metabolites of daunomycin (*i.e.*, daunomycinone and daunomycinol) or adriamycin (*i.e.*, adriamycinone and adriamycinol). These include three distinct procedures based upon silica columns: an ammonium acetate–isopropanol mobile phase[15,16], the ternary dichloromethane–methanol–ammonia–water system of Hulhoven and Desager[17,18] and the chloroform–methanol–glacial acetic acid–0.3 *M* magnesium chloride solvent system of Baurain *et al.*[19,20]. Others have utilised a phenyl stationary phase[13,21–23], the most effective being the tetrahydrofuran (THF)–ammonium formate gradient system of Andrews *et al.*[13], which resolved adriamycin and eight of its metabolites. Aliphatic side chain stationary phases have been studied systematically by Eksborg[24] (C_2, C_8 and C_{18}), with different organic modifiers, but restricted to phosphoric acid as an ionic modifier of the mobile phase. A C_{18} stationary phase has recently been used in pharmacokinetic studies of adriamycin and adriamycinol using acetonitrile–water–0.1 *M* phosphoric acid (37:60:3) as mobile phase[25].

One of the recent attempts to synthesise anthracycline drugs with greater activity has been based upon the correlation between their anti-cancer activity and their

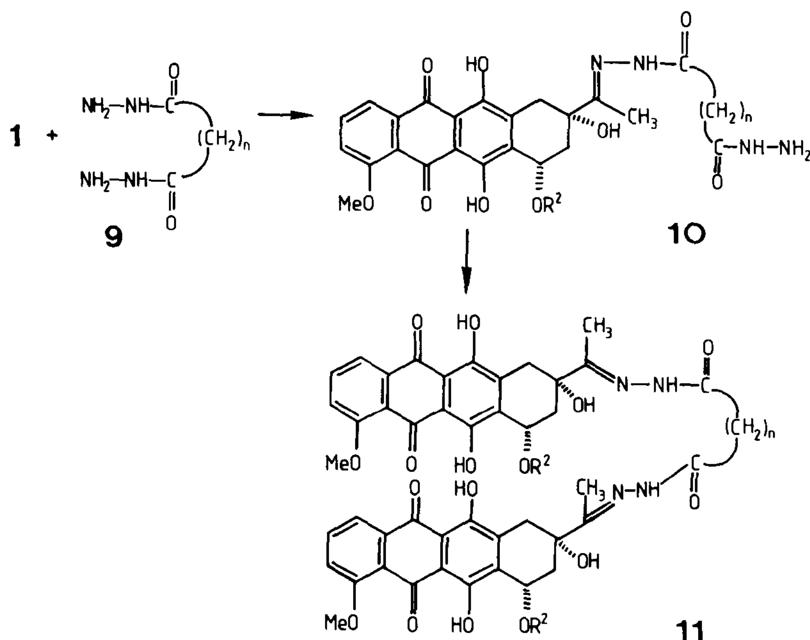

Fig. 2. Synthesis and structure of bis-daunomycin derivatives.

affinity for DNA[26], and utilises the concept of bis-intercalation[27–29]. A bis-intercalator has a potential association constant up to 10^{13} M^{-1} compared with 10^{6} M^{-1} for the parent drug at physiological ionic strengths[28]. There is therefore considerable interest in the synthesis of bis-intercalating bis-anthracyclines of the type synthesised by Henry and Tong[30] (Fig. 2), and hence, in separation and quantitation of individual components of intermediates (and contaminants) in such synthetic procedures, as well as semi-preparative methods for obtaining of the order of 10–100 mg amounts of highly purified products.

We sought an analytical HPLC system which had several specific characteristics. Firstly, that it offered good resolution of anthracyclines and result in baseline resolution to enable quantitation of synthetic derivatives of daunomycin and adriamycin, especially compounds of type 10 and 11 with molecular weights greater than 1000 daltons. Secondly, the process was required to be rapid, simple and inexpensive. Gradient elution procedures were therefore considered to be undesirable because o the column re-equilibration time involved, and relatively expensive HPLC grade solvents such as THF were to be avoided. Thirdly, it would be advantageous if the solvent–buffer system was amenable to only slight modification to enable its use in a semi-preparative mode (*i.e.* a completely volatile solvent–buffer system). We report here such an isocratic C_{18} reversed-phase system which offers good resolution of a range of anthracycline derivatives, is based on simple, inexpensive methanol–buffer mobile phase, resolves mono- and bis-derivatives of anthracyclines and has been adapted to semi-preparative work.

EXPERIMENTAL

Materials

Daunomycin, adriamycin and derivatives of these anthracyclines, were supplied by Farmitalia-Carlo Erba (Milan, Italy) and were used without further purification. All buffers and ionic modifiers used were of analytical reagent grade. The ion-pairing agent PIC-B8 was obtained from Waters Assoc. (Milford, MA, U.S.A.).

HPLC-grade solvents were obtained from Waters Assoc., while THF was obtained from Ajax Chemicals (Sydney, Australia). Ethanol and digol were of analytical reagent grade. Water used for all the HPLC solvent systems was obtained from a 4-bowl Milli-Q apparatus (Millipore, 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 Radial-Pak μBondapak C_{18}, and phenyl cartridges in a Z-module system, a 3.9 mm × 30 cm μBondapak C_{18} steel column and a 7.8 mm × 30 cm μBondapak C_{18} steel semi-preparation column, all from Waters Assoc. All the above columns were packed with 10-μm irregular particles. All analyses were carried out in an air conditioned room at 20°C.

Methods

The standard mixture of anthracyclines used consisted of a combination of the derivatives shown in Fig. 1. A small amount of each was dissolved in methanol (to yield approximately equal concentrations of 1 mg/ml) and their retention times ascertained individually for each column or solvent system used. The standard mixtures were then made by adding an increment of each derivative, and this solution was stored for up to two weeks at 4°C in the dark. The routine injection volume of this mixture was 20 μl.

Buffers were prepared daily with Milli-Q water and the pH adjusted, if required, with the appropriate salt or acid. They were then mixed with the organic solvent and degassed for 20 min immediately before use.

Synthesis of the bis-daunomycin pimelic hydrazone (11, $n = 5$) was performed in the dark at 20°C as described previously[30].

RESULTS AND DISCUSSION

A mixture of anthracycline derivative (Fig. 1), most of which are of physiological significance as metabolites of daunomycin or adriamycin[13], were selected as a standard for comparison of column capacity and selectivity of different HPLC elution conditions. Although the gradient system of Andrew *et al.*[13], utilising a phenyl column, gave reasonable separations of the compounds in the standard mixture when eluted isocratically, complete resolution was not possible. In fact, compounds 2 and 3 could not be resolved, and exhibited the same retention time (Fig. 3). Most importantly, this system also failed to resolve the mono-derivatives of daunomycin (10) from the bis-derivative (11). We therefore examined other columns and solvent systems in order to obtain improved selectivity of these compounds.

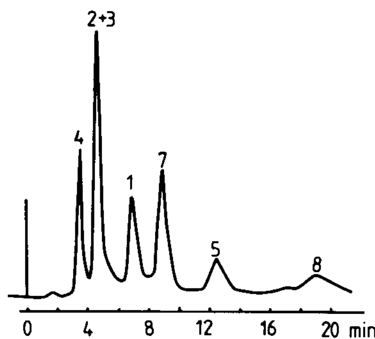

Fig. 3. Analytical HPLC trace of a mixture of anthracycline derivatives on a Waters Z-Pak phenyl column, eluted at 2 ml/min with THF–water (30:70) containing 0.1% ammonium formate buffer, pH 4.

More than 100 solvent systems were tested (using the standard mixture of derivatives) with Waters phenyl, C_{18}, CN and silica columns. The mobile phases included methanol–water, acetonitrile–water, ethyl acetate–methanol–water, ethanol–methylene chloride–water and digol–water. A variety of additives were included to allow for the charged nature of the drugs (and the residual polarity of the stationary phases). In unbuffered solvents, the inclusion of acids (those examined were formic, acetic, citric, glutaric and adipic acid) generally resulted in sharp peaks and good resolution. Bases such as pyridine, ammonia, triethylamine and morpholine resulted in complex chromatograms which were attributed to chemical reactions of the base with the anthracyclines. The buffered systems examined (tetrabutylammonium phosphate pH 4, ammonium phosphate pH 4, sodium phosphate pH 2–8, ammonium carbonate pH 8 and ammonium formate pH 4) were preferred since they could be prepared more reliably than the unbuffered systems, and generally yielded better resolution.

From the detailed work of Eksborg[24], it was clear that the selectivity of daunomycin–daunomycinol, daunomycinol–adriamycinol and adriamycin–adriamycinol would be similar for acetonitrile–water and methanol–water mobile phases. This was confirmed for the standard set of derivatives used in this work and therefore methanol was selected as the organic solvent of choice because of a cost advantage. With a methanol–water mobile phase, the best buffers found were tetrabutylammonium phosphate (Fig. 4a), ammonium acetate (Fig. 4b) and ammonium carbonate. For comparison, the profiles obtained with the ion-pairing agent octylsulphonic acid and with formic acid, are also included in Fig. 4c and d.

Several features of these profiles should be noted. Firstly, the more polar derivatives retain the same retention order, whereas the latter three more hydrophobic derivatives (compounds 5, 7 and 8) have different elution orders in each of the four systems shown. The buffer of choice therefore depends very much on the complexity of such hydrophobic derivatives present in any sample. Secondly, with ammonium acetate (and also ammonium carbonate which exhibits an essentially identical profile), the C_7 isomers of daunomycinol are apparent (Fig. 4b) and could readily be resolved with a less hydrophobic mobile phase. Because many laboratories possess Waters Z-Pak cartridges rather than the comparable stainless-steel column, it was of interest to determine if there were any significant differences between these two μBon-

dapak C_{18} columns. We show in Fig. 4e the trace obtained with the Z-Pak column, and note that the column efficiency (2σ method)[31] was 1500 plates/column as determined from component 7, compared with 3700 plates/column determined under identical experimental conditions and in the same manner for the stainless-steel col-

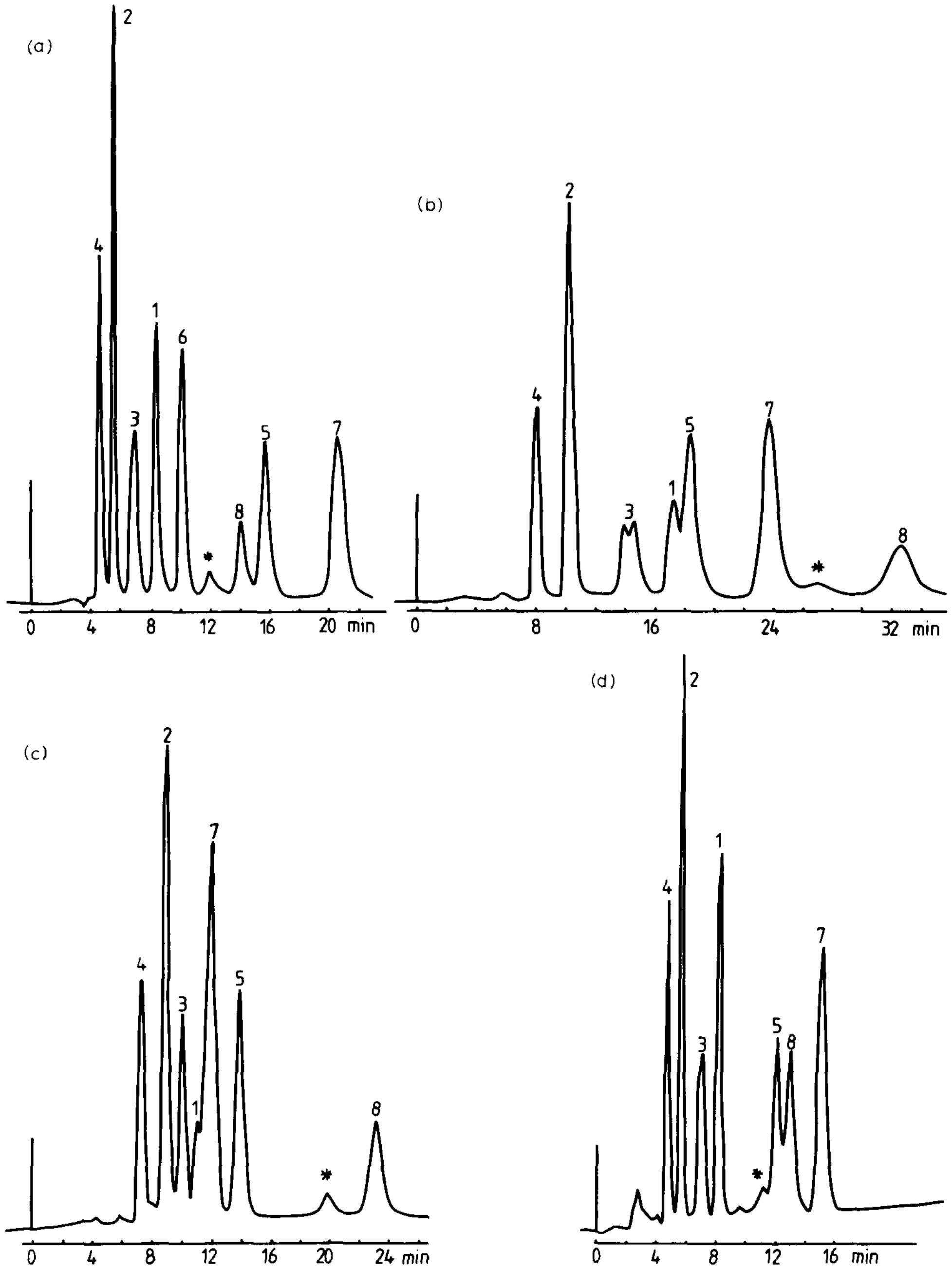

Fig. 4.

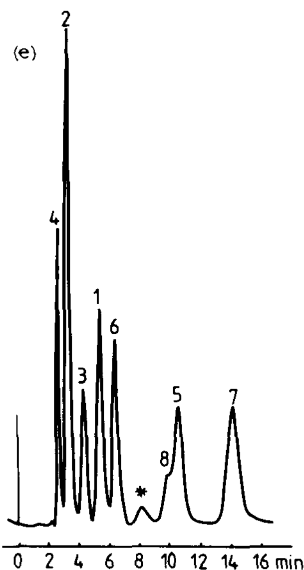

Fig. 4. Analytical HPLC traces of a mixture of anthracycline derivatives. (a) 1 ml/min, Waters stainless-steel μBondapak C_{18} column, methanol-water (65:35) with 10 mM tetrabutylammonium phosphate. (b) 1 ml/min, Waters stainless-steel μBondapak C_{18} column, methanol-water (65:35) with ammonium acetate (3%, w/v). (c) 1 ml/min, Waters stainless-steel μBondapak C_{18} column, methanol-water (70:30) with 5 mM Waters ion-pairing agent PIC-B8. (d) 1 ml/min, Waters stainless steel μBondapak C_{18} column, methanol-water (65:35) with 4% formic acid (98% solution). (e) 2 ml/min, Waters Z-pak μBondapak C_{18} column, methanol-water (65:35) with 10 mM tetrabutylammonium phosphate. The peak marked with an asterik is an unknown impurity present in 14-bromodaunomycin. The working pressure was *ca.* 2700 p.s.i. except in case (e) where it was *ca.* 1500 p.s.i.

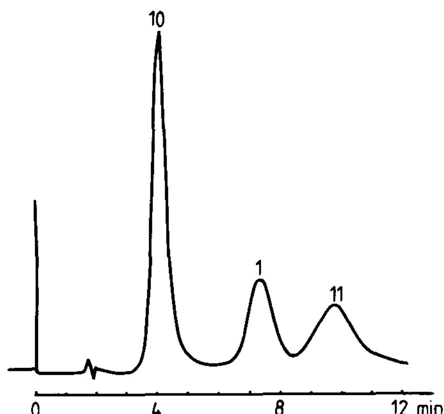

Fig. 5. Analytical HPLC trace of reaction mixture of daunomycin and four-fold excess of pimelic dihydrazide after standing at 20°C in the dark for four days. Elution conditions were the same as those for Fig. 4b. Peaks shown are daunomycin monohydrazide (10), daunomycin (1) and bis-daunomycin pimelic hydrazide (11).

umn (Fig. 4a). This expected loss of efficiency of the Z-Pak cartridge is most clearly exhibited by the lack of resolution of components 5 and 8, whereas baseline separation was obtained with the stainless-steel column.

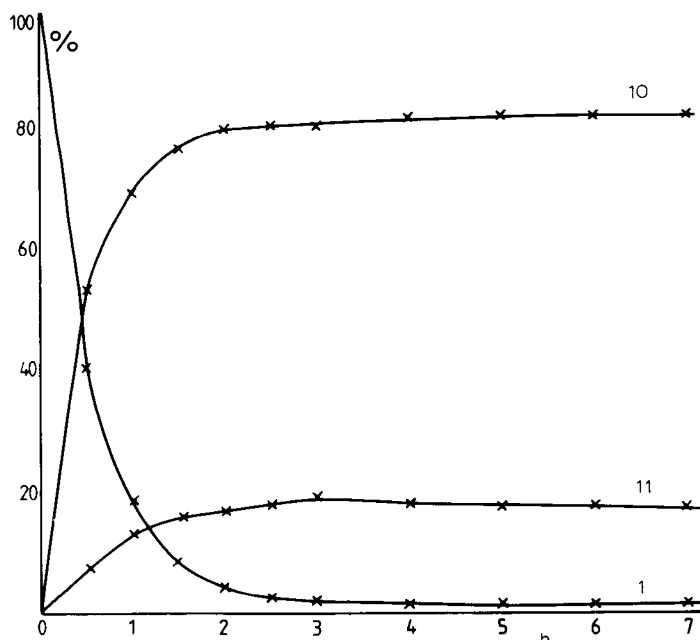

Fig. 6. Kinetics of formation of daunomycin monohydrazide (10) and bis-daunomycin pimelic hydrazide (11). The reaction conditions were as described in the legend to Fig. 5. The percentage composition of each component was determined from peak heights, assuming a common extinction coefficient.

Bis-anthracyclines

Pimelic dihydrazones of daunomycin (11, $n = 5$) were prepared by the method outlined by Henry and Tong[30]. The reaction mixture obtained in water with excess dihydrazide (9) was analysed using the C_{18} methanol–ammonium acetate system, and all three components (compounds 1, 10 and 11) were detected (Fig. 5). The resolution of these components therefore enables the kinetics of formation of the reaction to be monitored, under different reaction conditions, as well as determination of the equilibrium composition (Fig. 6). The reaction was essentially complete after 4 h at 21°C in the dark, but resulted in only a 17% yield of bis-anthracycline (Fig. 6). When using stoichiometric amounts of reactants in methanol (*i.e.*, duplicating the contributions of Henry and Tong[30]), 20% of mono-derivative (10), 1.7% of daunomycin and 78.3% of bis-daunomycin were detected, based on peak areas and assuming a common extinction coefficient for all components. These values are significantly different from the 100% yield of bis-daunomycin previously indicated by TLC analysis[30]. These results highlight the need for chromatographic systems which offer both good selectivity and good resolution for this class of compounds.

It is clear that the reaction proceeds by the formation of an intermediate, the daunomycin monohydrazide, and this subsequently reacts with an additional daunomycin moiety to yield the bis-daunomycin product. It is important to note that neither this 1:1 adduct has previously been detected with bis-anthracyclines[30], nor a similar 1:1 adduct with bis-acridines[32]. The solvent system demonstrated in the present work for resolving mono- and bis-anthracyclines could be expected to provide a basis for resolving bis-acridine reaction mixtures.

Semi-preparative HPLC

For semi-preparative work with bis-anthracyclines, we were restricted to those analytical HPLC conditions which offered good resolution and used a totally volatile solvent system. In addition, it was preferable to utilise a reversed-phase support, since these exhibit a capacity of up to ten times that of silica gels[33]. The methanol–ammonium acetate and methanol–ammonium carbonate systems described above therefore fitted these criteria. Since previous experience with drying anthracycline derivatives in the presence of ammonium acetate resulted in degradation of some compounds (presumably from exposure to residual acetic acid in the later stages of drying), methanol–ammonium carbonate was chosen. This system yielded complete resolution of all three components within 14 min when using methanol–ammonium carbonate (1.5%, w/v) (65:35, v/v). By altering the composition of the mobile phase, a wide range of resolution and selectivities are possible. The trace obtained with a Waters μBondapak C_{18} semi-preparative column (4 ml/min) was essentially identical to that obtained with the comparable analytical column (Fig. 5). The minimum time for complete separation to be achieved can therefore be estimated by selecting the resolution required to be commensurate with the amount of the particular drug in the mixture[34]. Because of the limited solubility of bis-anthracyclines (when linked by relatively insoluble inter-drug chains), and because this solubility is further decreased when the drug moieties are deprotonated on the column, sample loading is limited by this factor[34]. In this context, it is worth noting that large volumes of dilute solutions result in better efficiency than small volumes of more concentrated ones[33]. The maximum volume of sample that can be applied, while still retaining the required resolution, can be calculated from an equation derived (and confirmed experimentally) by Scott and Kucera[34].

The selectivity (α) of bis-derivative–daunomycin in methanol–ammonium carbonate (1.5% w/v) (65:35, v/v) was 1.3, and for the daunomycin–mono-derivative pair, $\alpha = 1.8$. These selectivities therefore permit some over-loading of the semi-preparative column without jeopardizing the extent of resolution of the bis-derivatives from the other reaction products[34].

We have previously used the completely volatile methanol–methylene chloride–water–ammonia solvent system of Hulhoven and Desager[17,18] with a semi-preparative silica column for separating complex reaction mixtures (up to ten products) of fluorinated derivatives of anthracyclines[35]. However, this system was unable to resolve the products obtained in the present work.

Of the many literature reports of the use of semi-preparative HPLC for purifying reactants or biological material, the vast majority utilize a simple volatile organic solvent–water system as mobile phase which presents no problem when drying the purified sample. The literature involving semi-preparative isolation of charged biological compounds is essentially non-existent except for the purification of peptides, which has required buffers and ionic modifiers in the mobile phase. Recently, good selectivity and recoveries have been reported with volatile buffers such as triethylammonium phosphate and formate (pH 3.5) and ammonium carbonate[36]. However, because of the effects of acid on the hydrazone linkage during drying of the sample, acidic buffers are unlikely to be useful for bis-anthracyclines of the type discussed in this paper.

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