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Investigations into the chromatographic behavior of a doxorubicin–peptide conjugate

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Abstract

HPLC impurity profile method development for a doxorubicin–heptapeptide conjugate included significant changes of the separation profile with diluent, eluent and pH. These separation variables were also temperature-dependent with a shift in retention from 35 to 45 °C. There was also a direct relationship of temperature with LC retention, and a pH minimum at 5.9. Atypical dependence of the impurity profile on diluent at a k' of 18 led to further investigation. A large change in retention by several minutes was a function of both the organic eluent composition and temperature between 15 and 30 °C. Several Van't Hoff temperature studies from 5 to 65 °C on several column types resulted in non-linear plots. Analysis of the molecular subunits suggested that the peptide portion of the analyte influenced the non-linear retention behavior. The stationary phase type was not a significant factor causing non-linearity. Circular dichroism–temperature studies indicated a notable transition in ellipticity for the amine regions (198–202 nm) that occurred between 39 and 44 °C. This transition temperature range coincided with the results of the Van't Hoff analysis, between 35 and 44 °C, to indicate that these effects were not primarily stationary phase induced.

Keywords: Temperature effects; Thermodynamic parameters; Doxorubicin; Peptides

1. Introduction

Prodrugs have been shown to increase drug efficacy through molecular design and modeling [1]. Carrier-linked prodrugs can alter molecular solubility making the prodrug more water soluble [2] or more lipophilic [3]. Carriers may be modified to improve absorption and distribution. This will not only augment efficacy but also eradicate many side effects associated with poor delivery to the intended site [4]. Carrier-linked prodrugs may influence more sophisticated advanced metabolic conjugation in the systemic circulation to maximize dose response for slow and prolonged release [5]. Prodrugs also may reduce formulation problems and improve in vivo stability, thus affecting bioavailability [6,7].

Proteins and protein-linked drug molecules have been a great challenge for solution structural elucidation [8,9] and chromatographic optimization [10]. The structure of a protein may contain multiple primary and secondary amines, zwitterionic charge, regions of intramolecular H-bonding that are solution dependent [11], and may also be dependent on state

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variables such as temperature and pressure [12]. When a peptide polymer is used as a transport molecule for drug delivery [13] additional questions arise regarding its solution behavior. Typically peptides rely on structure–activity relationships to bind specifically to an active receptor site. If the presence of the attached drug molecule in solution offsets this structural recognition process, the result is not as expected. Understanding the physical properties of protein-linked carriers as a function of diluent and temperature can assist in understanding their separation behavior.

The characterization of proteins is further complicated by a strong dependence of structure on mixing effects. Side chain interactions and internal bond formations in aqueous environments can intensify at higher temperatures as more of the hydrophobic portions of the analyte are exposed [14]. Understanding the conformational changes of oligomeric (short-sequence) peptides in different lipophilic environments offers some insight as to the choice of solvent changes to induce various conformations [15]. Oligopeptides at the core of the sequence have the potential to induce a more global change in molecular conformation [16]. Therefore, small amino acid sequences can provide a clearer understanding of the behavior that occurs in larger protein sequences. Similarly, what occurs in macromolecular proteins can also be traceable to the subunits or small amino acid sequences. For this reason, peptides and the small peptide subunits were previously used to understand atypical behavior such as shifts in the analyte retention with temperature change and oligomer chain length [17–19]. Other small homo-chiral amino acid sequences can self-associate independent of the column but rather as a function of solvating environment [20]. Baldwin [21] developed a liquid hydrocarbon model to predict contributions of hydrophobic interactions of aliphatic amines to the thermodynamics of protein folding. This may result in an analyte being less retained at separation temperature extremes but most retained at intermediate separation temperatures (35–45 °C). Transitions in analyte retention with temperature are not limited to proteins but can also be extended to aromatic molecules for benzene and phenol derivatives, as shown by Hammers and Verschoor [22]. Larger polyaromatic hydrocarbons, with both fused and separated rings, displayed interesting temperature and solvation affects that altered RPLC selectivity [23,24].

Several theories have been postulated to account for such behavior. One theory is that the stationary phase undergoes morphological changes with temperature during a separation, altering the stationary phase binding sites accessible to small organic molecules [25,26]. Formation of such cavities in the stationary phase may alter the hydrophobic properties of the phase, resulting in different analyte interactions with either the bonded phase or the silica linker [27]. Proteins can alter conformational properties to obscure true impurities of the protein carrier [28]. Increasing the bonded phase density makes these temperature–retention transitions more pronounced. Deviations from a linear dependence of retention on temperature may also be associated with the phase type used for the separation of large planar aromatic molecule, such as monomeric C_{18} vs. polymeric C_{18}. Such phases may undergo a structural change at a defined and distinct critical temperature, influencing the retention of analytes [29]. A second theory attributes thermal retention abnormalities observed for peptides and proteins more to the mobile phase composition. With this theory, stationary phase pores of different sizes are created as a function of the H-donor ability of the mobile phase. Such changes in the protic nature of the mobile phase may influence the relative system phase ratio [30,31]. A third theory suggests that unique multisite interactions on-column may lead to secondary or tertiary peptide structures, which in turn affect analyte retention properties. Stationary phase-induced conformational changes of proteins have been reported [32,33]. Solubility and stationary-mobile phase permeability must also be considered. The underlying issue is that these theories may all be valid for a given analyte and a particular separation, but no single theory accounts for all of these observations.

In this work, interesting reversed-phase liquid chromatographic behavior was observed as a function of mobile phase composition, analyte diluent, and separation temperature for a peptide-linked doxorubicin conjugate (**1**) during methods development, which was linked to the unique structure of

(**1**). Molecule (**1**) has a seven amino acid peptide, linked to deliver doxorubicin, a demonstrated chemotherapy agent, to prostate cells with cancer [34,35]. Furthermore, through chromatographic analysis of available structural subunits of (**1**), it was demonstrated that the atypical behavior observed was related to the heptapeptide portion of the molecule. The structures of (**1**) and its related subunits: the heptapeptide (**2**), doxorubicin (**3**), adriamycinone (**4**), 9-fluorenylmethyl (Fm)-protected heptapeptide (**5**), the Fm-tripeptide (**6**), and the tetrapeptide (**7**) are provided in Fig. 1.

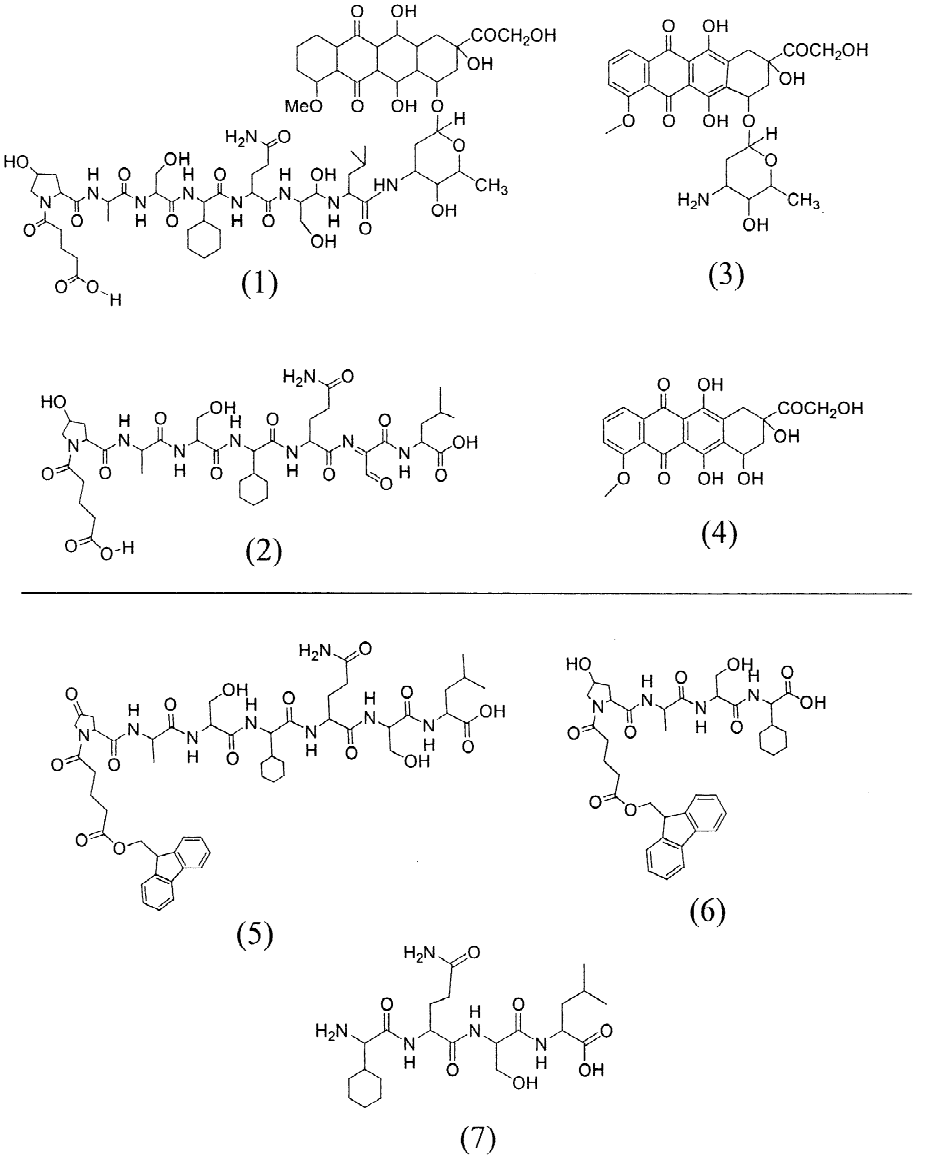

Fig. 1. The doxorubicin heptapeptide conjugate (**1**) with subunits of (**2**) the heptapeptide, (**3**) doxorubicin and (**4**) adriamycinone. The true subunit terminus in all cases is hydrogen, if not specified. The 9-fluorenylmethyl (Fm)-substituted peptide, Fm-heptapeptide (**5**) is shown with subunits of the Fm-tripeptide (**6**) and the tetrapeptide (**7**).

2. Experimental

2.1. Materials

N-(Glutary–Hyp–Ala–Ser–cyclohexylglysyl–Gln –Ser–Leu)–doxorubicin (**1**; Fig. 1), was obtained from the Process Research Department of Merck Research Labs. (Rahway, NJ, USA). The heptapeptide (**2**) and all 9-fluorenylmethyl protected subunits (**5**, **6**, and **7**) were obtained from Sicor Labs. (Milan, Italy). Doxorubicin–HCl (**3**) was obtained from Mercian Labs. (Fort Lee, NJ, USA). Adriamycinone (**4**) was obtained from Spectrum (New Brunswick, NJ, USA).

2.2. Solvents

Acetonitrile, ethyl acetate and ammonium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Both 2,2,2-trifluoroethanol and piperidine were obtained from Aldrich (Milwaukee, WI, USA), and methanol and tetrahydrofuran were received from EMS (Gibbstown, NJ, USA). Ultra-purified water was generated with a Hydro Picotech System (Garfield, NJ, USA) and sodium hypochlorite was received from Clorox (Oakland, CA, USA).

2.3. Instrumentation

All HPLC experiments were performed with an HP1100 liquid chromatograph from Agilent (Wilmington, DE, USA). A Discovery C_8 column was purchased from Supelco (Bellefonte, PA, USA), the Advantage Phenyl column was purchased from Analytical Sales and Services (Mahwah, NJ, USA) and the XDB-C_{18} was purchased from Mac-Mod Analytical (Chadds Ford, PA, USA). Chromatograms were acquired using Turbochrom Client/Server Software Version 6.1 for Microsoft NT (Perkin-Elmer, Norwalk, CT, USA). All concentrations of (**1**) through (**7**) were measured by direct mass using an Analytical Balance AE-186 and an AT261 Delta Range (Mettler, Toledo, OH, USA). The rotary evaporator was a Buchi R-124 (Fisher Scientific, Nutley, NJ, USA) attached to a laboratory-supplied vacuum source.

2.4. Chromatographic conditions

Two gradient methods were used for the impurity profiles. Gradient method A used 0.1% (v/v) phosphoric acid adjusted to pH 7.1 with ammonium hydroxide (aqueous) and acetonitrile (organic), UV detection at 215 nm, 1.0 ml/min at 30 °C column temperature. The aqueous organic gradient was held at 10% organic for 5 min, then steadily increased at 0.8% min to 24% organic by 22 min and then held for 8 min before increasing at 1.4% organic/min to 65 min before 10 min of re-equilibration. Gradient method B used the same column, flow and detection as above at 15 °C with mobile phase 0.1% (v/v) acetic acid adjusted to pH 5.8 with ammonium hydroxide (aq.) and acetonitrile (organic). The aqueous–organic gradient was held for 5 min at 20% organic phase, then increased by ~0.6%/min to 30% organic, and finally organic content was increased at a rate of 1.1%/min to 70%.

Temperature studies for (**1**) and its subunits used an Agilent XDB-C_{18} phase (250×4.6 mm, 5 μm particles) with an isocratic mobile phase at [~5 m*M* aqueous ammonium acetate (pH 5.8)]–acetonitrile (60:40, v/v), 1.0 ml/min flow-rate. Column temperature was varied between 5 and 65 °C in increments of 5 °C with analyte detection at 215 nm. An analysis pH close to the peptide zwitterionic p*I* was selected to stabilize the doxorubicin linker by minimizing the potential of the keto portion of the molecule to form water adducts at low pH[1] [38]. The mobile phase was also used as a sample diluent to eliminate potential chromatographic artifacts due to mixing effects. All chromatographic analyses used the same conditions, unless otherwise noted, with the exception of the temperature studies for the heptapeptide which used a Symmetry Shield C_{18} column to maximize peptide retention.

2.5. Circular dichroism spectral experiments

A Model J715 Jasco Spectropolarimeter with J700 Windows Temperature/Wavelength software for Version 1.30.00 was used for all circular dichroism

[1]NMR evidence for doxorubicin in deuterated solvents indicate shifts C-8 and C-10 carbon centers the nearest neighbours for the ester moiety that suggests this is the place of water addition [39].

(CD) measurements with an external Biolabs (Surrey, Canada) temperature bath using the J-715 Control Driver 1995 (Jasco, Easton, MD, USA). The CD spectra of 0.2 mg/ml solutions of (**1**) at 30 °C in trifluoroethanol (TFE), 5% TFE in methanol and acetonitrile–water (50:50, v/v) were acquired to mimic diluents used in chromatographic gradient studies.

2.6. Deprotection of the Fm-heptapeptide

The heptapeptide used for chromatographic studies was obtained from the deprotection of a 9-fluorenylmethyl protected heptapeptide using 15% (v/v) piperidine in dimethylformamide (DMF) with nitrogen sparge, followed by rotary evaporation, and finally extraction of the 9-fluorenylmethyl protecting group into ethyl acetate as per previously published procedures [36,37].

3. Results and discussion

3.1. Evaluation of chromatographic parameters

Initial attempts at chromatographic characterization of (**1**) at separation temperatures above ambient in acetonitrile–water mobile phase and diluent systems (gradient A) led to a large amount of peak fronting (Fig. 2a). LC–MS analysis confirmed the on-column conversion of (**1**) into a water adduct (M+18) as a function of the diluent water content and analysis temperature [38]. Reported NMR studies of doxorubicin in deuterated acetonitrile–water systems indicate that the terminal ketone of doxorubicin can associate with water, forming an M+18 adduct as was observed chromatographically in our laboratories for the analysis of (**1**) [39].

Modifications to the mobile phase gradient (gradient B) and reduction in analysis temperature minimized fronting, as seen in Fig. 2b. However, one can also see in Fig. 2b that the choice of diluent greatly impacts the ability to resolve minor impurities near the parent compound despite significant column residence time ($k' \sim 18$), suggesting that a small amount of trifluoroethanol in a strong H-donating diluent is essential for resolution. The

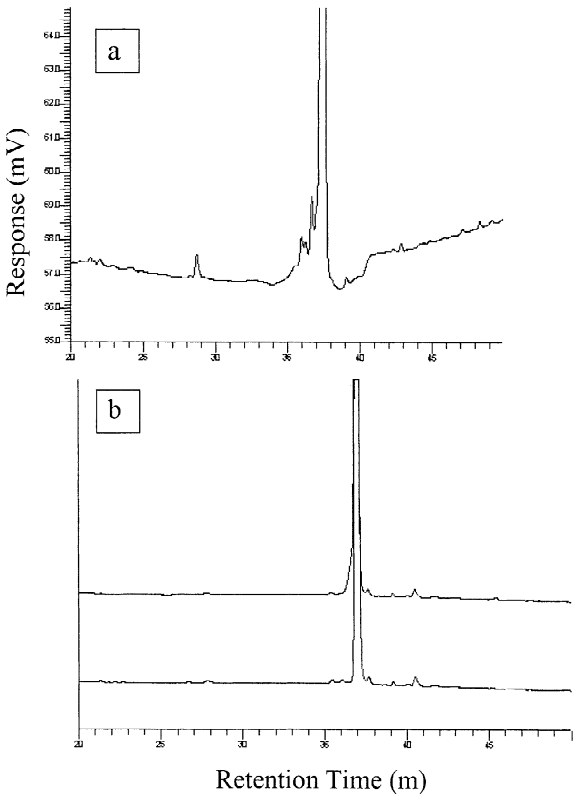

Fig. 2. (a) The HPLC impurity profile for the ~0.2 mg/ml doxorubicin conjugate (retention 38–40 min) using a Zorbax Eclipse XDB-C_{18} (250×4.6 mm, 5 μm) column at 30 °C using gradient method A. (b) The same column at 15 °C as above was used with gradient method B. Diluent changes for ~0.2 mg/ml solutions using 100% trifluoroethanol (b, top) and 5% trifluoroethanol in methanol (b, bottom). m = minutes.

solubility of (**1**) in both trifluoroethanol and 50% (v/v) aqueous acetonitrile mixtures is greater than 50 mg/g, and about 0.4 mg/g in methanol. Increased ratios of amphiphilic solvents such as trifluoroethanol increase solubility of (**1**), but may induce small degrees of protein order, such as self-association for the hydrophobic faces of the molecule [40]. Altering this solvent environment can alter the relative charge affinity affecting the molecules assembling tendency [41]. Based on the large differences in polarity between the peptide and anthracycline portions of (**1**), the alkyl bonded phase may also influence analyte ordering during the separation [42].

The retention and peak shape of weak acids, bases,

and zwitterions may be controlled by ion suppression, typically achieved through pH adjustment. It also is known that charge effects related to the pH for individual amino acids can promote structure for a molecule [43]. A minimum isocratic retention time was observed for (**1**) at a pH corresponding to the average isoelectric point (p*I*) of the individual amino acids in the heptapeptide molecule (5.9). This suggested that the ionizable peptide portion (**2**) of (**1**), rather than the anthracycline sugar portions (**3**) or (**4**), dominated the relative retention behavior of the molecule, as one would expect.

Since (**1**) has only a seven amino acids sequence, it is unlikely to form significant degrees of secondary structure [44]. However, small changes in peptide–peptide or peptide–anthracycline interactions in solution may occur and influence chromatographic behavior. The potential affect of solvent environment on (**1**) was demonstrated by CD spectroscopy as shown in Fig. 3. Although the CD spectra indicate very little evidence of helicity, there were subtle changes in the peak minima at 201–205 nm. The solvent component can bind to C=O and NH groups of the peptide bonds not involved in intramolecular H-bonding [45]. Similar comparisons of the CD spectra of (**2**) (not shown) to (**1**) in these solutions indicated that the anthracycline significantly alters the solution behavior of the molecule. This is also evident in the different solubility of (**2**) compared to (**1**).

Fig. 4 shows the relationship between the retention and mobile phase composition behavior of (**1**). Similar retention characteristics were observed at 10 and 30 °C as a function of organic mobile phase composition, but significantly different behavior was noted at 20 °C. These changes and considerable peak broadening that occurred were the result of dramatic mixing effects between the mobile phase and the 40% acetonitrile aqueous diluent. Consequently, it is evident that the retention behavior of (**1**) in Fig. 4 was affected by both the temperature and organic mobile phase composition at 20 °C, to cause a notable change in retention at higher organic compositions.

A final interesting chromatographic observation was the direct proportionality of retention time of (**1**) with column temperature. Typically this relationship is inversely proportional for most organic molecules.

These analyses suggested that the unique structure of (**1**) plays a major role in the chromatographic behavior observed, and that the temperature may be a critical underlying dependent variable. These re-

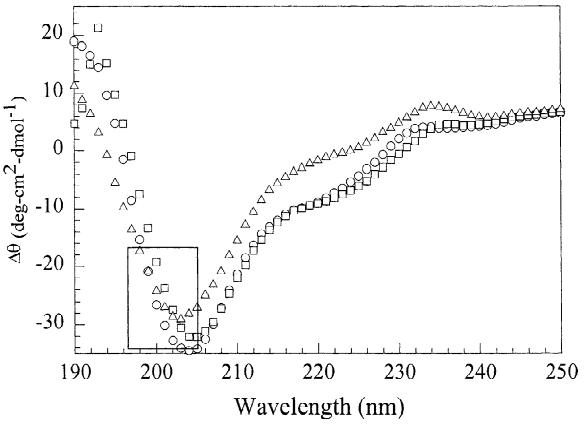

Fig. 3. Circular dichroism ellipticity change at 200 nm for the doxorubicin conjugate and 198 nm for heptapeptide subunit-representing changes to the *cis*/*trans*-amine regions (indicated with box) of the random coil as a function of diluent. Trifluoroethanol (circles), 5% trifluoroethanol in methanol (squares), 50% acetonitrile in water (triangles).

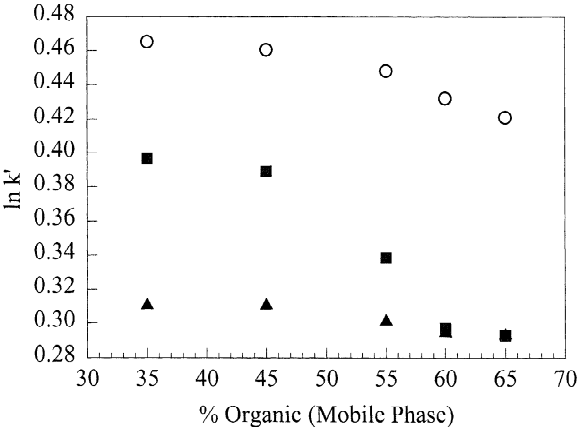

Fig. 4. The effect of eluent composition on the retention behavior of the doxorubicin conjugate for the isocratic separation with ammonium acetate buffers (pH 5)–acetonitrile mixtures of 35, 45, 55, 60 and 65% acetonitrile with an XDB-C_{18} column at 10, 20, and 30 °C. The diluent was 40% acetonitrile in water for a 0.4-mg/ml solution.

sults encouraged further investigations into the thermodynamics of the separation in an attempt to elucidate the retention mechanism(s).

3.2. Van't Hoff analysis

The Van't Hoff equation (1), a well-established thermodynamic relationship, allows for the calculation of the separation equilibria at different temperatures. A typical Van't Hoff analysis allows one to indirectly relate the magnitude of the separation–association enthalpy, one value of enthalpy (ΔH) in kJ/mol, for a given temperature region via the following linear relationship:

$$\ln k' = -\Delta H/RT + \Delta S/R + \ln \phi \quad (1)$$

where $R = 8.314$ J/K-mol, ΔS is the entropy and ϕ is the phase ratio or relative volume of stationary phase to mobile phase.

Temperature studies, using Eq. (1), were completed using C_8, C_{18}, and phenyl stationary phases, as shown in Fig. 5. Similarly non-linear Van't Hoff plots were observed for (**1**) on all three columns. Such behavior has previously been noted for proteins [33,34]. The higher column bonded phase density for the C_8 column, with a 4.6-μm particle size, appeared to have greater retention properties and be slightly less curved relative to the phenyl and C_{18} columns, both with 5 μm particles. Since however, changing the stationary phase type did not significantly change the non-linear nature of the temperature dependence, it may be inferred that this behavior is significantly influenced by thermally induced changes to the analyte during the separation, and less to do with the functional group used in the stationary phase. This behavior is dissimilar to previous observations reported for the separation of the regio-isomers of rizatriptan benzoate, where the primary factor influencing the non-linearity of Van't Hoff plots appeared to be the choice of stationary phase [46]. In order to characterize the entire temperature range that was investigated, a non-linear fit to the Van't Hoff data was judged to be most appropriate. The XDB column was arbitrarily selected for subsequent Van't Hoff analyses unless otherwise noted.

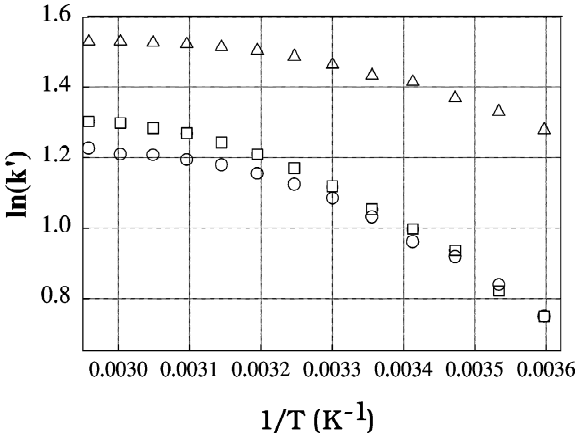

Fig. 5. Van't Hoff curvilinear plots natural log of the capacity factor with several column types: a Discovery C_8, 250×4.6 mm, 4.3 μm (triangles), a Lancer phenyl, 250×4.6 mm, 5 μm (circle) and an Zorbax XDB-C_{18} 250×4.6 mm, 5 μm (squares) using an isocratic method at 60% ammonium acetate buffer pH 5.8 and 40% acetonitrile at column temperatures varying from 5 to 65 °C in 5 °C increments.

3.3. Non-linear Van't Hoff analysis

Curved Van't Hoff results, that include a peak maxima or minima, may be fit with two separate linear portions by omitting the inflection region as shown by Waters et al. [47]. This data treatment can simplify calculations for the temperature extremes. However, for truly curved plots, linear forced regions may also lead to a disparity for the calculated enthalpy, at the middle temperatures of the separation. This is especially true when curvature occurs over several temperature values. For better interpretation of change in the curved regions of the Van't Hoff plots, a quadratic fit was applied.

Quadratic Van't Hoff fits as explained previously by Haidalher et al. [48], and more recently by Hearn and Zhao [49], exploit Kirchoff's relations. A temperature-dependent ΔH, ΔS and ϕ yield non-linear plots, the result of changes to the enthalpic and entropic heat capacity. The phase ratio (ϕ) over the temperature region used here (5–60 °C) can, for these comparison purposes, be considered unity. The relative magnitude of ϕ in this region can change at most by 10% between 5 and 50 °C—to result in a change for the estimated heat capacity to be no higher than 3% [50]. Changes in heat capacity of oligo-peptides can be directly associated to the

properties of amino acids making up the molecule [51]. This treatment of the data allows for the indirect determination of association enthalpy, entropy, heat capacity and free energy. The magnitude of change for these terms allows one to interpret potential changes in the equilibria. The quadratic fits allow for temperature dependent values for enthalpy, and entropy as shown with the assumption that the phase ratio is unity.

$$\text{Quadratic:} \quad \ln k' = a/T^2 + b/T + c + \ln \phi \tag{2}$$

$$\text{Enthalpy:} \quad \Delta H = -R[b + (2a/T)] \tag{3}$$

$$\text{Entropy:} \quad \Delta S = R\left[c - \left(a/T^2\right)\right] \tag{4}$$

$$\text{Heat capacity:} \quad \Delta C_p = \left(2Ra/T^2\right) \tag{5}$$

$$\text{Free energy:} \quad \Delta G = \Delta H - T\,\Delta S \tag{6}$$

The values for the second-order coefficient, *a*, and the first-order coefficient, *b* are empirical constants, derived from the slope changes in the fit. The intercept value represents the natural log of the phase ratio and a third term *c*. These derivations allow the calculation of the molar enthalpy and entropy of association for the analyte relating to the degree of molecular interaction and order with the stationary phase, respectively. The molar heat capacity term represents the second-order changes in slope with temperature, the result of Kirchoff's relations. This second order change is usually suggestive of the hydrophobic effect of the analyte, the ability of apolar groups to associate in avoidance of energetically unfavorable interactions with water [52]. The free energy associated with the overall separation in Eq. (6) relates the relative dependencies of the enthalpy and entropy to the overall separation energy.

3.4. Thermodynamic evaluation of the doxorubicin conjugate and the subunits

Non-linear Van't Hoff relationships Eq. (2) were used to analyze the data collected. Van't Hoff plots were constructed for (**1**) and its available subunits (**2**)–(**5**) as shown in Fig. 6. The subunits were selected in an attempt to identify the portions of the

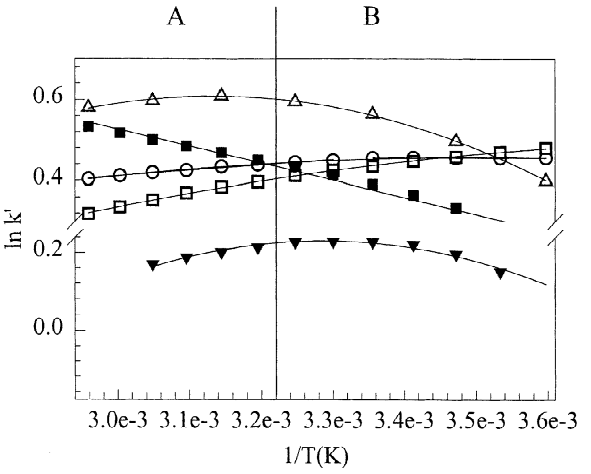

Fig. 6. Non-linear Van't Hoff comparison of (**1**, open triangle) and its subunits (**3**, open circles), (**4**, open squares), (**5**, inverted triangle), and (tetra, solid squares). The quadratic fit using the same isocratic method as described by Eq. (2). Regions about the inflection point are divided by a generally enthalpic region (A) and a generally entropic region (B).

molecule most responsible for the retention behavior observed. Fig. 6 was divided into two regions around the approximate inflection point range, in the curved plots from 35 to 45 °C for (**1**) and 30 to 35 °C for (**2**). Region A (~60 to 38 °C) was governed by a generally enthalpic separation mechanism; and region B (~38 to 5 °C) was governed by a generally entropic mechanism. It appears that the key contributor to the retention of (**1**) on the XDB-C_{18} column is the anthracycline portion of the molecule, as evidenced by the fairly linear and enthalpically driven behavior of compounds (**3**) and (**4**). However, the entropically driven retention behavior of (**1**) in region B seems to be dominated by contributions from the peptide portion. The individual subunits of the heptapeptide side chain (**5**), (**6**) and (**7**) were similarly treated via non-linear Van't Hoff analyses to further define the role of the individual subunits in the thermodynamic behavior of (**1**) in Fig. 7. All three peptidyl fragments have similar degrees of curvature with a similar inflection point range at 30–35 °C, relative to that of 35–45 °C for (**1**).

The overall curvature of the retention data for (**1**) appears to be greatly influenced by the heptapeptide portion of the molecule (**2**) as well as the subunits. The similarity of the quadratic fit using Eq. (2) for

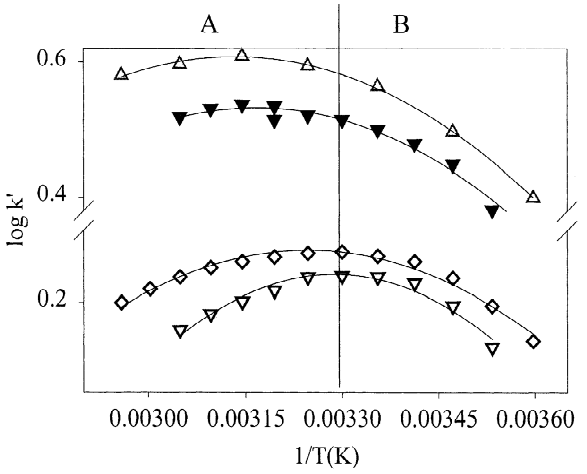

Fig. 7. Non-linear Van't Hoff comparison of (**5**) and its subunits to (**1**) with the same isocratic method as previously described. Regions A (generally enthalpic) and B (generally entropic) about the inflections points with solid inverted triangle=(**5**), open triangle=(**1**), inverted open triangles=(**2**), and diamonds=(**6**).

compounds (**1**) and (**2**) shown in Table 1 supports this general observation.

When the enthalpy of a separation shows temperature dependence, this results in a non-zero value for the heat capacity. As Vailaya and Horváth [50] indicated, a non-zero heat capacity suggests another event occurs during the separation that results in a temperature-dependent enthalpy and entropy. Concave Van't Hoff plots indicate that there also exists a change in the non-zero heat capacity with temperature. Plots of ΔC_p vs. T were made over the range of 278–330 K from the non-linear Van't Hoff data and Eq. (5). Table 2 compares the relative magnitude of the slopes ($\Delta\Delta C_p/\Delta T$) and intercepts ($\Delta C_p$ at 278 K)

Table 2
Subunit slope and heat capacity change related to Van't Hoff curvature

Analyte	Slope/1000	ΔC_p at 278 K
(**3**) Doxorubicin	0.17	−0.044
(**4**) Adriamycinone	0.55	−0.089
(**7**) Tetrapeptide	0.78	−0.14
(**2**) Heptapeptide	1.5	−0.25
(**5**) Fm-heptapeptide	2.7	−0.48
(**6**) Fm-tripeptide	2.2	−0.37
(**1**) Doxorubicin conjugate	2.5	−0.48

in the regions of interest. All fits were linear with a correlation of 0.98 or better. The fact that slopes of (**3**) and (**4**) are closer to zero suggests negligible molecular reorientation with temperature, hence a single solution equilibrium process occurs. The large slopes shown for (**1**) and (**2**) indicate the magnitude of curvature in the Van't Hoff plots. The large negative heat capacity values at 278 K become closer to zero (defined by larger positive slopes) at higher temperature, which usually occurs when non-polar substances transfer to water [21]. This change in ΔC_p with temperature is characteristic of competing solution equilibria occurring during the separation.

Significant heat capacity changes for transfer of large proteins into water may be a result of the ordering of water around the non-polar molecule (entropic effect), a change in the non-polar surface area, or protein-folding behavior. Frank and Evans [53] long ago introduced the change in molar heat capacity with the concept of ''iceberg formation'' to relate to the increased ordering of water around the non-polar analyte accompanied by an increase in the degree of hydrogen bonding. They indicated that a

Table 1
Correlation and empirical constants from the quadratic fits (**1**) and the subunits

Analyte	a (second-order slope/10^6)	b (first-order slope/10^3)	c (Intercept+ln ϕ)	r^2
Doxorubicin conjugate	−2.2	14.0	−21.0	0.9999
Heptapeptide	−1.2	7.8	−13.0	0.9503
Doxorubicin	−1.8	1.3	−1.7	0.9960
Adriamycinone	−0.44	3.5	−5.6	0.9993
Tetrapeptide	−0.67	3.5	−3.1	0.9975
Fm-tripeptide	−1.7	11.0	−18.0	0.9718
Fm-heptapeptide	−2.3	14.8	−22.0	0.9983

negative enthalpy change accompanied by an increase in ordering or drop in entropy with temperature relates to a change in the structure. Nemethy and Scheraga [14] related these changes to aliphatic and aromatic associations of isolated side chains. These structural perturbations do not require major conformational change, but rather subtle changes in the secondary amine environment result in considerable changes in analyte retention and solution behavior. Taylor et al. [54] discuss a similar trend with increased temperature which relates the solution behavior to the stability of the backbone of a helix. Based on the previous CD spectra, the molecule does not have a preferred conformation or helix. However, regardless of the macromolecular conformation or lack thereof, the same ordering effect can occur as a function of increasing temperature, resulting in a more negative enthalpy of hydration. What is remarkable is that these effects are observed with a peptide as small as seven and three amino acid units, to result in an apparent entropic effect.

The common property shared by (**1**), (**2**), (**5**) and (**6**) was the combination of a polar amino acid side chain tethered to a large planar aromatic moiety. This combination suggests competing equilibria exists between the peptide portion and the separation phase, when the peptide portion is tethered to a rigid backbone such as the doxorubicin or 9-fluorenylmethyl molecule. In theory, these competing equilibria contain an enthalpic component, for the mass transfer during the linear reversed-phase separation, and an entropic component, of the intramolecular reorientation of the peptide chain with the planar aromatic molecule. The entropic regions of the non-linear Van't Hoff plots are perhaps areas when the molecular orientation equilibria are greater than the separation equilibria. The intramolecular reorientation may be governed by the local interaction of the peptide with the non-polar planar aromatic moiety. The greater this association becomes, the smaller the associations of the non-polar aromatic portion with the column, and thus the shorter the retention. For this reason, (**5**) and (**6**) have the greatest magnitude slopes ($\Delta\Delta C_p/\Delta T$) and intercepts ($\Delta C_p$ at 278 K) and thus the most non-linear temperature dependence. On the other hand, compound (**7**) without both a glutaryl end group and an aromatic tether has the smallest heat capacity change and magnitude of the peptide containing analytes. If these changes to the structure actually occur in solution, we should see solution differences with temperature by CD spectroscopy, in the same solvent environments used on-column.

3.5. CD temperature studies

CD temperature studies were performed at the composition of the isocratic method, using the precise mobile-phase ratio of elution. The data in Fig. 8 indicate the dependence of the molar-ellipticity differences ($\ln \Delta\theta$) for the 201 nm region for the temperature range from 10 to 80 °C. In the temperature region from 39 to 44 °C, significant slope changes occur, which coincide with the inflection for the Van't Hoff plots of (**1**) in Fig. 6 at ~35–45 °C. These results support that the changes observed in the chromatography of (**1**) were strongly related to the affects of both temperature and the solvent on the unique structural features of the analyte.

Preliminary ^{1}H NMR–temperature studies in deuterated acetonitrile (40%) in pH 5.8 buffer indicated a chemical shift change for the center C_8 of the doxorubicin molecule. Since there were no observed alterations with temperature for the keto-group of doxorubicin and no modification in the leucine or

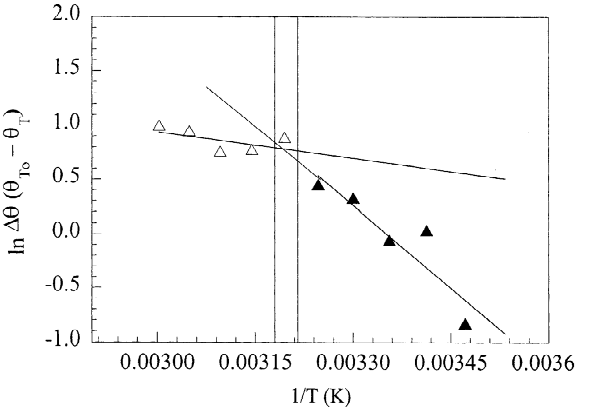

Fig. 8. (A) Circular dichroism ellipticity difference vs. temperature for the doxorubicin conjugate in 60% pH 5.8 (~5 nm) ammonium acetate buffer and 40% acetonitrile—the elution composition used for the Van't Hoff analyses plot of the ln of the change vs. $1/T$. The highlighted region represents 317 K (44 °C) to 312.5 K (39.5 °C) the estimated region of transition in ellipticity at 201 nm.

alanine, or in the glutaryl end group chemical shifts, the center of the molecule around the serine–cyclohexyl–glycine–glutamine sub-sequence is suspected of taking part in the association. Future work planned includes additional chromatographic studies, as well as NMR, Raman, and CD spectroscopy to further assess the nature of the conformational changes of (**1**) during reversed-phase separation.

4. Conclusion

The interesting separation behavior for (**1**) as a function of pH, mobile phase composition and temperature in reverse phase separations can be attributed to its unique structure. Other chromatographic anomalies in the impurity profile such as the M+18 water adduct the diluent dependence at k' over 18, and the entropic separation behavior were attributed to (**2**) the peptide portion of the molecule. The determination of the inflection points in the curvilinear Van't Hoff plots of (**1**) and its subunits suggest the presence of alternate temperature-dependent equilibria. The solution CD studies showed a strong dependence on diluent type. CD temperature studies indicate that this particular molecular behavior is not significantly influenced by the solution environment and temperature. Chromatographic temperature studies with (**1**) and its subunits indicate the peptide region has the most influence on the observed non-linear retention behavior. Intramolecular interactions between the peptide chain and the nonpolar planar aromatic molecule, to which it is tethered, are believed to be primarily responsible for the observed atypical retention behavior.

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