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Determination of minor conformational changes of a doxorubicin–peptide conjugate under chromatographic conditions

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Abstract

Thermodynamic analysis of the reversed-phase retention behavior of a doxorubicin–peptide conjugate demonstrated that the degree of non-linearity observed in Van't Hoff plots was impacted by mobile phase acetonitrile content over the 25–38% acetonitrile (v/v) range tested. Small decreases in the non-polar surface area of the doxorubicin–peptide conjugate as a function of temperature were estimated from these data using linear solvent strength relationships, suggesting that the retention behavior may be the result of minor analyte conformational changes during the chromatographic experiment. This hypothesis was supported via circular dichroism (CD), Raman and ^{1}H NMR spectroscopic studies of the doxorubicin–peptide conjugate in selected chromatographic mobile phase compositions. The CD and Raman data indicated small changes to the apparent analyte microenvironment as a function of temperature and bulk solvent environment, while ^{1}H NMR studies specifically demonstrated the environmental sensitivity of protons on three non-polar peptide residues and the proximal aromatic region of the analyte. Together, these data suggest that minor changes to the conformational order of the essentially random structure of the doxorubicin–peptide conjugate are sufficient to impact chromatographic performance.

Keywords: Thermodynamic parameters; Mobile phase composition; Linear solvent strength relationships; Doxorubicin; Peptides

1. Introduction

Recently, the reversed-phase liquid chromatographic behavior of a doxorubicin–peptide conjugate was reported [1]. A numbered structure of the doxorubicin–peptide conjugate is provided in Fig. 1, with a hydroxyproline–alanine–serine–cyclohexylglycine–glutamine–serine–leucine amino acid sequence (with a glutaryl end group) in the heptapeptide sidechain. In this previous work, non-linear Van't Hoff plots were obtained for both the doxorubicin–peptide conjugate and its fluorenylmethyl protected-peptide sidechain in a acetonitrile–aqueous ammonium acetate (pH 5.8) (40:60) mobile phase on C_8, C_{18}, and phenyl bonded phases, with an inflection point ~40 °C between the two linear regions of the plot. Many reports of similar non-linear Van't Hoff plots have been published for reversed-phase separations and attributed to either changes in retention mechanism or changes in stationary phase

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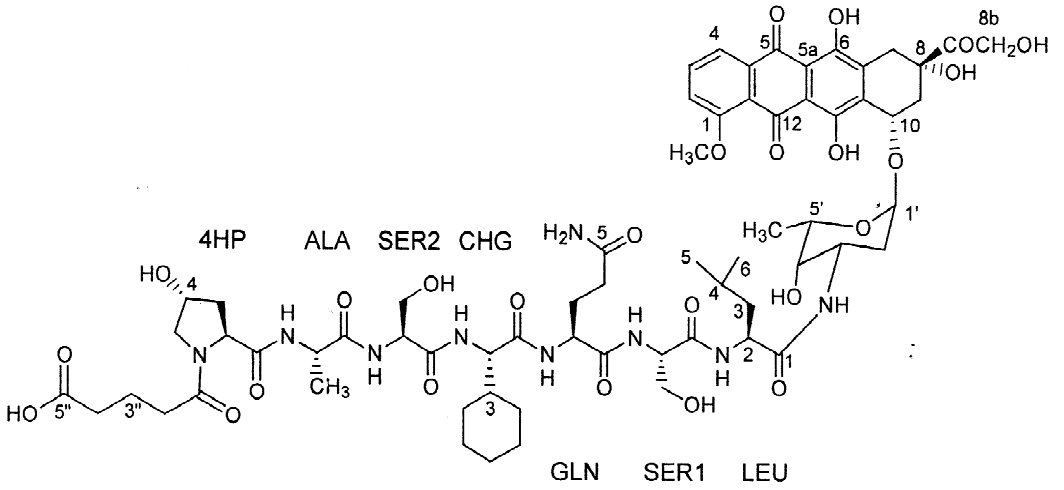

Fig. 1. Numbered structure of the doxorubicin–peptide conjugate.

structure over the temperature range studied [2]. Commonly, the inflection point in such plots of chromatographic data is near the phase transition temperature of neat octadecane liquid (28–30 °C) if the effect is attributed to modifications in stationary phase organization with temperature. Alkyl chain reordering as a function of temperature has also recently been confirmed via Raman spectroscopy [3–5]. However, an inflection temperature at ~30 °C is not consistent with our observations for the doxorubicin–peptide conjugate [1], suggesting that the non-linear retention behavior observed was not due to alkyl chain reordering effects.

In the same previous work, solution circular dichroism (CD) data were collected, which indicated the doxorubicin–peptide conjugate was predominantly a random coil in solution. Further, CD spectra were obtained as a function of temperature and an inflection point at ~40 °C was observed, suggesting that conformational reordering of the analyte was related to the chromatographic observations. This was supported by additional chromatographic data from which linear Van't Hoff plots were constructed for the more rigid adriamycinone and doxorubicin structural subunits of the doxorubicin–peptide conjugate on the same stationary phase. These data suggest the significant role the heptapeptide tether of the molecule plays during conformational changes.

Molecular spectroscopic techniques such as ultraviolet (UV), fluorescence, and CD spectroscopy are amongst the most commonly used techniques to assess both analyte conformation and conformational changes in solution, however, these techniques are limited to probing electronic transitions in analytes possessing chromophores. Vibrational spectroscopic techniques, such as Raman and infrared spectroscopies, allow one to detect analyte conformational changes as small frequency shifts of characteristic local modes, however these spectral changes are not generally unique to conformational types [6–8]. Evaluation of the spectral band shape is also incorporated into the interpretation of vibrational circular dichroism data (VCD) from macromolecules, lending added insight into conformational type [9]. Nuclear magnetic resonance (NMR) and X-ray crystallography techniques are among the only ones to potentially provide specific determination of absolute molecular structure.

While these spectroscopic techniques have been mostly employed to assess significant changes to the secondary structure of larger molecules, they may also be used to analyze more subtle changes in smaller peptide fragments. Absence of secondary structure for the doxorubicin–peptide conjugate is not unexpected from both the short length of the peptide chain and the differing propensities of the adjacent individual amino acid residues in the heptapeptide to form α-helices, β-sheet, or β-bend conformations [10]. Similarly, a heptapeptide with lysine–serine–glutamic acid–glutamic acid–

glutamine–leucine–alanine (KSEEQLA) sequence was also confirmed to be in random coil conformations in aqueous trifluoroethanol and acetonitrile solutions [11]. Sleep peptide, a nonapeptide with a tryptophan–alanine–glycine–glycine–aspartic acid–alanine–serine–glycine–glutamic acid (WAGGDASGE) amino acid sequence, remains unordered even in surfactant environments [12]. However, angiotensin I, angiotensin II, and insulin B are 10-7 amino acid peptides, respectively, each with β-forming potential at surfactant concentrations below the critical micellar concentration [13,14]. Additionally, several inactive elastin hexapeptides were determined to be random coils, while other hexapeptide sequences, active in matrix metalloproteinase 1 production, have propensities to form a β-turn [15]. Such results highlight the importance of amino acid sequence in determining the potential for formation of secondary structure.

Various approaches have been taken to further understand the relationship between the spectroscopically-determined conformation of analytes and their observed reversed-phase liquid chromatographic performance. Molecular simulations were used to model the docking of Bombesin (14 amino acid sequence) to reversed-phase sorbents, permitting assessment of the orientation of hydrophobic moieties with the stationary phase [16]. Conformational changes of proteins and peptides upon interaction with a reversed-phase sorbent have also been experimentally monitored under gradient elution chromatographic conditions with empirical models related to linear solvent strength (LSS) theory [17–20]:

$$\log \bar{k} = \log k_0 - S\bar{\varphi} \quad (1)$$

where $\bar{k}$, $\bar{\varphi}$, $\log k_0$ represent the mean capacity factor, mean volume fraction of organic solvent in the mobile phase, and mean capacity factor in fully aqueous environments, respectively. The magnitude of S is proportional to the hydrophobic contact area of a peptide and thus should modulate as a function of temperature when significant changes in conformation (often changes in secondary structure) occur during the reversed-phase experiment.

In this manuscript, additional chromatographic retention data for the doxorubicin–peptide conjugate will be presented as a function of both mobile phase acetonitrile content and column temperature to demonstrate the collective effects of these variables. Using some of the spectroscopic and empirical chromatographic tools outlined in the introduction, efforts will be made to establish the relationship between the chromatographic observations and specific conformational perturbations of the analyte during the chromatographic experiment. Our previous work has established the doxorubicin–peptide conjugate as a generally random structure, hence potential conformational changes are likely minor and insignificant factors in the biological function of the subject molecule. However, these changes appear to have significant impact on the chromatographic behavior of the analyte, hence developing an understanding of these effects is of value.

2. Experimental

2.1. Materials

N-(Glutaryl–Hyp–Ala–Ser–Cyclohexylglycyl–Gln–Ser–Leu)–doxorubicin (1) was obtained from the Process Research Department of Merck Research Labs. (Rahway, NJ, USA).

Acetonitrile and ammonium acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and HPLC-grade water was generated with a Hydro Picotech System (Garfield, NJ, USA). Deuterated ammonium hydroxide, deuterated acetic acid, deuterium hydroxide, and deuterated acetonitrile for NMR studies were purchased from Aldrich (Milwaukee, WI, USA).

2.2. Chromatographic measurements

All HPLC experiments were performed with a complete Agilent HP1100 liquid chromatograph with ultraviolet detection at 215 nm (Wilmington, DE, USA). Thermal retention studies for the doxorubicin–peptide conjugate over a 10–70 °C range were performed with 5 m*M* aqueous ammonium acetate (pH 5.8)–acetonitrile mobile phases on Inertsil ODS2 columns (MetaChem Technologies, Torrance, CA, USA) over isocratic mobile phase compositions ranging from 25 to 38% (v/v) acetonitrile. Sample

concentrations of approximately 0.2 mg/ml doxorubicin–peptide conjugate were used for these experiments. Two different length ODS2 columns were used to maintain an acceptable capacity factor (k') for the analyte over this wide isocratic mobile phase composition range. A 4.6×50 mm, 5 μm ODS2 column was used for retention studies using 25, 27, 28.5 and 30% (v/v) acetonitrile mobile phases and a 250×4.6 mm, 5 μm ODS2 column was used for 31, 32, 34, 36 and 38% (v/v) acetonitrile mobile phases.

2.3. CD spectroscopic experiments

A Model J715 Jasco Spectropolarimeter with J700 Windows Temperature/Wavelength software for Version 1.30.00 was used for all CD measurements with an external Biolabs (Surrey, Canada) temperature bath using the J-715 Control Driver 1995 (Jasco, Easton, MD, USA). To examine the relationship of the conformation of the doxorubicin–peptide conjugate to its observed chromatographic behavior, CD spectra were acquired in selected mobile phase compositions at 20, 35 and 60 °C. Solutions were prepared in 25, 30 and 38% (v/v) acetonitrile in 5 m*M* aqueous ammonium acetate (pH 5.8) diluents at 0.2 mg/ml concentrations to mimic concentrations used in chromatographic experiments.

2.4. Raman spectroscopic experiments

A Kaiser HoloProbe process Raman analyzer (Kaiser Optical Systems, Ann Arbor, MI, USA), equipped with a fiber-optic probe and a 785 nm diode laser to minimize sample fluorescence, was used for the direct acquisition of Raman spectra via the HoloGrams software package. These data were then transferred into ASCII format for final data analysis via functions programmed in Matlab (The MathWorks, Natick, MA, USA). The Raman probe was directly inserted into a sealed three-necked flask, which was immersed in a water bath with feedback temperature control.

Solutions of the doxorubicin–peptide conjugate were prepared in 25, 30 and 38% acetonitrile (v/v) in 5 m*M* aqueous ammonium acetate (pH 5.8) diluents at 8.6–9.6 mg/ml concentrations to achieve required sensitivity, or nearly 50-fold higher than the chromatographic concentrations. However, chromatographic retention times were constant over the 0.2–5 mg/ml range in these solvent systems, suggesting that the doxorubicin–peptide conjugate does not undergo significant intermolecular self-association and therefore the Raman data at these concentrations are representative of the chromatographic data. These samples were added to the above apparatus and analyzed independently, with constant stirring, in the above apparatus at 20, 35 and 60 °C. Five Raman scans, each with 6 s exposure, were averaged as one spectrum. The resulting spectra for each solution of the doxorubicin–peptide conjugate were blank–corrected with the corresponding solvent composition/temperature combination used for the sample under identical data acquisition conditions.

2.5. NMR spectroscopic experiments

Approximate 3 mg/ml doxorubicin–peptide conjugate solutions were prepared using all deuterated solvents for ^{1}H NMR analysis to mimic the 25 and 38% (v/v) acetonitrile in 5 m*M* aqueous ammonium acetate (pH 5.8) mobile phases used during the chromatographic studies. Each solution was analyzed at 20, 35 and 60 °C with a Bruker Avance 600 MHz NMR system and all spectra were referenced to $CH^{2}H_{2}CN$. Additional two-dimensional ^{1}H NMR experiments were conducted to facilitate in peak assignments.

3. Results and discussion

3.1. Van't Hoff studies

As an extension of previous work, retention studies for the doxorubicin–peptide conjugate as a function of both mobile phase composition and temperature were completed using the Van't Hoff relationship [Eq. (2)].

$$\ln k' = -\Delta H/RT + \Delta S/R + \ln \Phi \quad (2)$$

Plots of $\ln k'$ versus $1/T$ were constructed over a 10–70 °C temperature range and isocratic mobile phase compositions ranging from 25 to 38% (v/v) acetonitrile content in aqueous ammonium acetate buffer. Fig. 2 provides the data for only the 25% and 38% (v/v) acetonitrile mobile phases, with linear

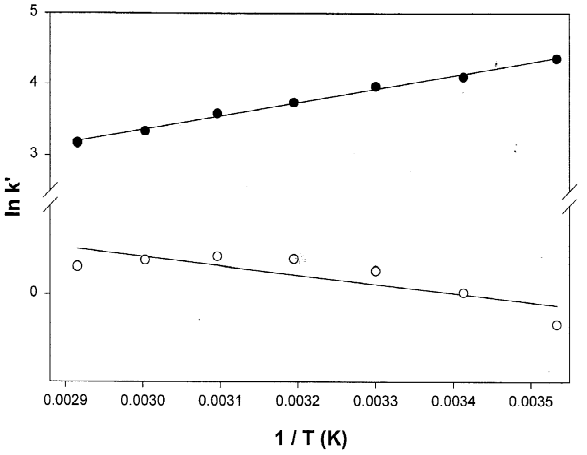

Fig. 2. Linear Van't Hoff plots for the doxorubicin–peptide conjugate in 25% and 38% (v/v) acetonitrile mobile phases.

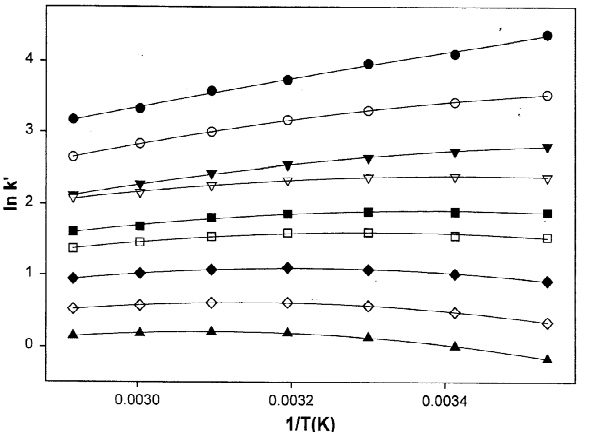

Fig. 3. Quadratic Van't Hoff plots for the doxorubicin–peptide conjugate in 25–38% (v/v) acetonitrile mobile phases. ●=25%; ○=27%; ▼=28.5%; ▽=30%; ■=31%; □=32%; ♦=34%; ◇=36%; ▲=38%.

regression fits for each. For 25% (v/v) acetonitrile, the doxorubicin–peptide conjugate generated a linear Van't Hoff plot ($r^2=0.994$) with positive slope, indicating retention was well-described by the classical equation (enthalpically-driven). However, as the acetonitrile content of the mobile phase is increased to 38% (v/v), the plot becomes non-linear ($r^2=0.674$). These thermodynamic retention results are quite similar to those previously obtained for the doxorubicin–peptide conjugate at 40% (v/v) acetonitrile [1].

Increased conformational order in peptides is often observed with increasing organic solvent content and may be further enhanced by use of surfactants [14]. If minor enhancements in doxorubicin–peptide conjugate conformational ordering generally occurs as acetonitrile content increases within the range tested, it follows that the retention data acquired at higher organic content should be more sensitive to temperature due to disruptions in this ordering. Therefore, the Van't Hoff plot created in 38% (v/v) acetonitrile mobile phase has the potential to exhibit more non-linearity than that obtained in 25% (v/v) acetonitrile, as was observed in this work.

To better model the non-linear retention data above, a quadratic equation was used [Eq. (3)], where a, b, and c are empirical constants and Φ is the mobile/stationary phase ratio [21,22]. Fig. 3 provides the quadratic fits for all retention data from 25 to 38% (v/v) acetonitrile mobile phase contents, where r^2 ranged from 0.970 to 0.999 for the various curve fits. From this relationship, the entropic, enthalpic, and heat capacity changes in kJ mol^{-1} K^{-1} at 293, 313, and 333°K were estimated via Eqs. (4)–(6), respectively, and summarized in Table 1. These temperatures represent a data point near the common inversion temperature in all Van't Hoff plots (~40 °C), as well as a data point on either side of the inversion temperature (20 and 60 °C):

$$\ln k' = a/T^2 + b/T + c + \ln \Phi \quad (3)$$

$$\Delta H = -R\,[b + (2a/T)] \quad (4)$$

$$\Delta S = R[c - (a/T^2)] \quad (5)$$

$$\Delta C_p = (2Ra/T^2) \quad (6)$$

Generally speaking, the enthalpic interactions of the doxorubicin–peptide conjugate with the stationary phase are more negative, hence more favorable, at lower acetonitrile content as would be expected. However, at 34–38% acetonitrile contents, enthalpies become positive at both 20 and 40 °C and the entropic term dominates. Correspondingly, the heat capacity shows an increasing temperature dependence with mobile phase acetonitrile content. Such phenomena have been previously attributed to minor changes in analyte conformation as a result of

Table 1
Thermodynamic parameters calculated for the doxorubicin–peptide conjugate as a function of volume percent acetonitrile in the mobile phase

Parameter	Acetonitrile (%, v/v)								
	25	27	28.5	30	31	32	34	36	38
ΔH (293 K)	−14.36	−7.87	−5.31	−0.27	0.59	2.72	5.85	8.19	10.57
ΔH (313 K)	−15.66	−11.31	−8.67	−3.72	−3.23	−1.46	1.04	3.12	5.06
ΔH (333 K)	−17.57	−16.34	−13.59	−8.77	−8.82	−7.59	−6.02	−4.31	−3.03
ΔS (293 K)	3920	10331	10095	10368	11475	12574	14467	15245	16594
ΔS (313 K)	3920	10331	10095	10368	11475	12574	14467	15245	16594
ΔS (333 K)	3920	10331	10095	10368	11475	12574	14467	15245	16594
ΔC_p (293 K)	−0.09	−0.24	−0.24	−0.24	−0.27	−0.29	−0.34	−0.36	−0.39
ΔC_p (313 K)	−0.08	−0.22	−0.21	−0.22	−0.24	−0.27	−0.31	−0.32	−0.35
ΔC_p (333 K)	−0.07	−0.19	−0.18	−0.19	−0.21	−0.23	−0.26	−0.27	−0.30

increased ordering of water about non-polar moieties of a molecule, resulting in decreased available contact area for hydrophobic interactions with the stationary phase [23,24].

3.2. LSS studies

The retention data collected to prepare Fig. 3 were used to populate the empirical LSS model described by Eq. (1). Fig. 4 is a plot of S and log k_0 as a function of the mobile phase volume fraction of acetonitrile. These plots demonstrate a general monotonic decrease in analyte/stationary phase interactive contact area with increasing temperature during partitioning. Similar behavior was previously demonstrated for similar small peptides (penta- and heptapeptides), as well as small peptide fragments from tryptic digests [11,25,26]. Upon closer examination, there is an inflection point in both the S and log k_0 plots at approximately the same temperature (35 °C) as the inflection points were noted in the Van't Hoff plots for the doxorubicin–peptide conjugate (Fig. 3). However, one would anticipate disruption of an analyte's hydration sphere with increasing temperature, which would lead to increased analyte non-polar contact area, counter to present observations. These data suggest that minor doxorubicin–peptide conjugate conformational changes occur either in solution and/or during partitioning with the stationary phase, which serve to reduce the accessibility of key non-polar functional groups for binding.

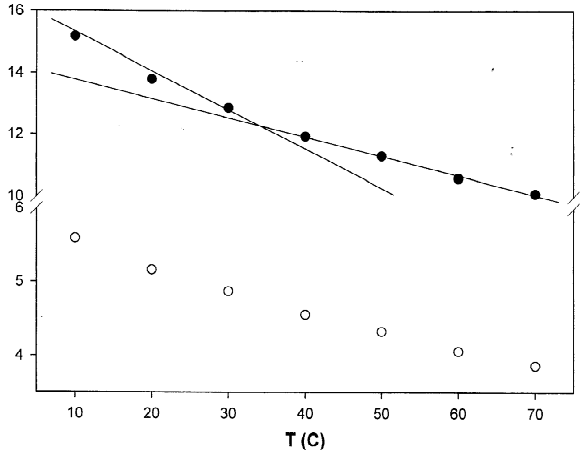

Fig. 4. Plot of LSS parameters S (●) and log k_0 (○) as a function of separation temperature for the doxorubicin–peptide conjugate.

3.3. CD spectroscopic studies

The previous thermal CD spectroscopic studies of the doxorubicin–peptide conjugate at 40% (v/v) acetonitrile in aqueous ammonium acetate buffer were extended to include 25, 30, and 38% (v/v) acetonitrile solvent systems at 20, 35, 60 °C (Fig. 5). The general observation may be made that the effects of temperature at 25% (v/v) acetonitrile are smaller than those at higher acetonitrile contents, suggesting that the conformation of the analyte is less impacted by temperature changes at this composition. This observation is consistent with the chromatographic

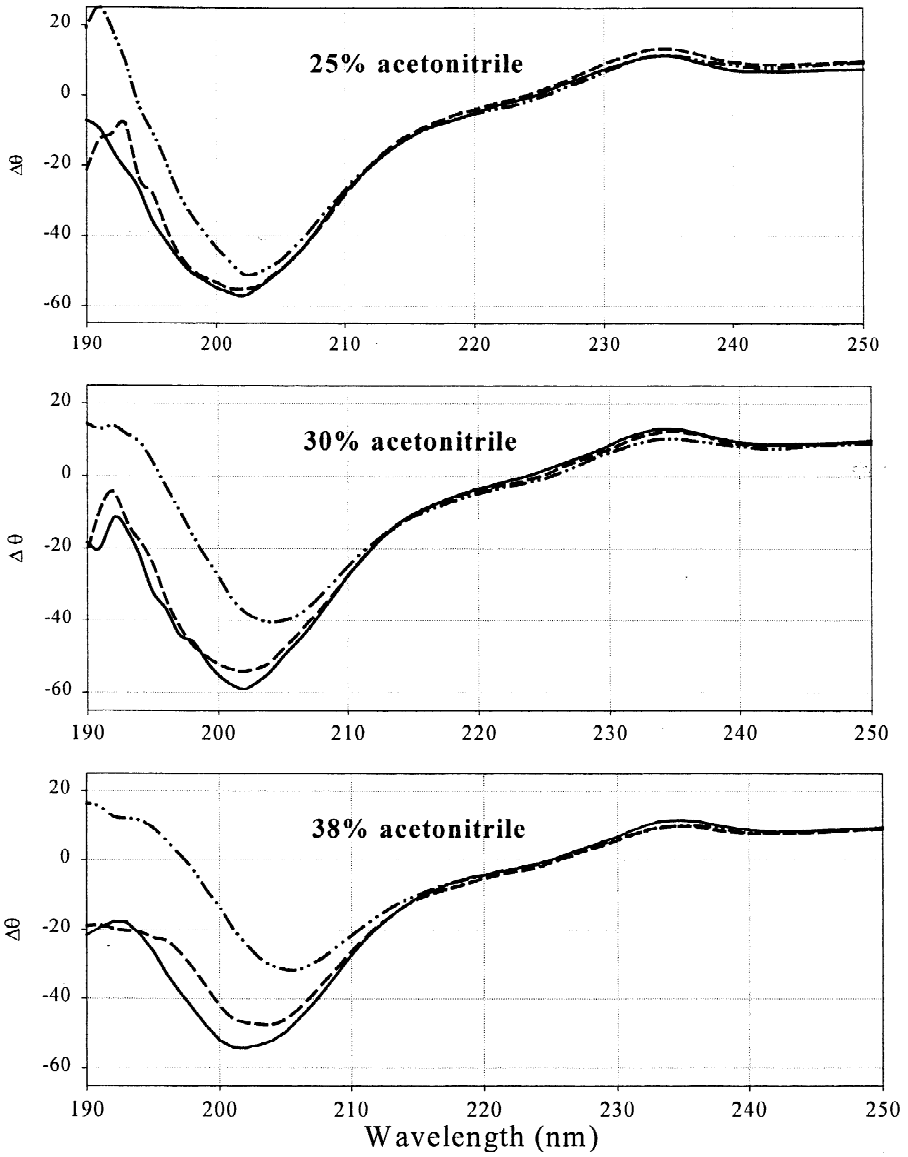

Fig. 5. CD spectra of the doxorubicin–peptide conjugate as a function of solvent composition and temperature. Solid line = 20 °C, dashed line = 35 °C; dashed and dotted line = 60 °C.

retention results presented in Fig. 3, in which lower acetonitrile content produced linear plots and higher acetonitrile contents produced non-linear plots, due to proposed conformational changes. All spectra in Fig. 5 have a characteristic negative dip at 200–205 nm, consistent with $\pi-\pi^*$ transitions in randomly ordered peptides with no significant secondary structure [1,15]. Small bathochromic shifts in this peak are observed with increasing temperature, which is further enhanced at higher organic content (lower polarity). This is counter to expectations, since small bathochromic shifts are expected from such chromophores in more polar environments [27]. These results suggest that the apparent polarity of the doxorubicin–peptide conjugate microenvironment is different than that of the bulk solution at higher

acetonitrile content. Further, since the CD signal is predominantly generated by the absorbance of the fused-ring system of the analyte, this suggests that changes in microenvironmental polarity occur in this portion of the molecule.

3.4. Raman spectroscopic studies

Raman spectroscopic analysis of doxorubicin solutions was performed in the same environments and at the same temperatures as employed in the previous CD studies (see Fig. 6). The most meaningful change in the spectra is a small shift of the Amide III peak (AIII) at approximately 1375 cm^{-1} region. AIII is often the most structure-sensitive amide band which originates from the in-phase combination of NH bending and CN stretching modes [28]. Shifts in the position of the AIII peak to higher frequencies are observed in Fig. 6 as acetonitrile content is increased, which is consistent with previous reports for peptides due to increased hydrogen bonding between water and the amide proton, as would be expected in matrices containing more organic modifier [29]. A second general observation made from these data is that decreasing temperature in each solvent matrix also resulted in AIII shifts to higher frequencies, which is consistent with the anticipated effects of temperature on hydrogen bonding. Others have suggested that AIII shifts to higher frequencies may also occur due to stabilization of the amide resonance structure via enhanced CN double bond character in more polar environments [30]. In the present study, AIII shifts to higher frequencies are observed with decreasing bulk environment polarity, suggesting that the peptide portion of the analyte has a somewhat more polar microenvironment than that of the bulk solution. A similar conclusion was suggested for the aromatic region of the doxorubicin–peptide conjugate via CD spectroscopy. Coupling these spectroscopic data with the chromatographic data, it appears that the conformation of the analyte is influenced by the degree of hydration of the flexible peptide tether as well as subsequent intramolecular interactions of the peptide with the aromatic portion of molecule.

3.5. NMR spectroscopic studies

Through analysis of the effect of both temperature and solvent composition on the ^{1}H NMR spectrum of the doxorubicin–peptide conjugate, an attempt was made to identify the specific regions of the molecule involved in the proposed conformational changes. Only the chemical shifts (δ, ppm) of the protons on C_2, C_3, C_4, C_7, C_{10}, and the methoxy functional group on C_1 of the fused ring system, as well as protons on the methyl group of leucine and protons of the glycine and cyclohexylglycine residues, were observed to be influenced by temperature and composition (Table 2). A general trend of approximately 0.1–0.2 ppm deshielding was observed for these protons as a function of increasing analysis temperature in both 25 and 38% (v/v) deuterated acetonitrile solvent systems (see the Experimental Section for details of solution preparation). Similar degrees of deshielding were observed for these protons at each analysis temperature in both 38% (v/v) and 25% (v/v) deuterated acetonitrile solvent systems.

Shifts in ^{1}H NMR signals as a function of solvent composition and temperature are possible in polar analytes due to preferred orientation of solvent molecules about an analyte or molecular associations, respectively [31]. These results suggest that interactions are possible between these three affected peptide residues and their most proximal regions of the fused ring system, which may reduce the non-polar contact area of the aromatic portion of the molecule, as was suggested from the chromatographic data presented. Unfortunately, we were unable to confirm this hypothesis by nuclear Overhauser effect experiments. It is also important to note that the affected peptide residues are the most non-polar in the molecule, hence such conformational changes may also inhibit the accessibility of these groups to the stationary phase during partitioning which would affect reversed-phase liquid chromatographic performance.

4. Conclusions

The balance of chromatographic and spectroscopic evidence presented suggest that the doxorubicin–peptide conjugate undergoes minor conformational changes as a function of temperature and solvent composition. These changes involve differences in hydration of molecule's peptide tether, as well as

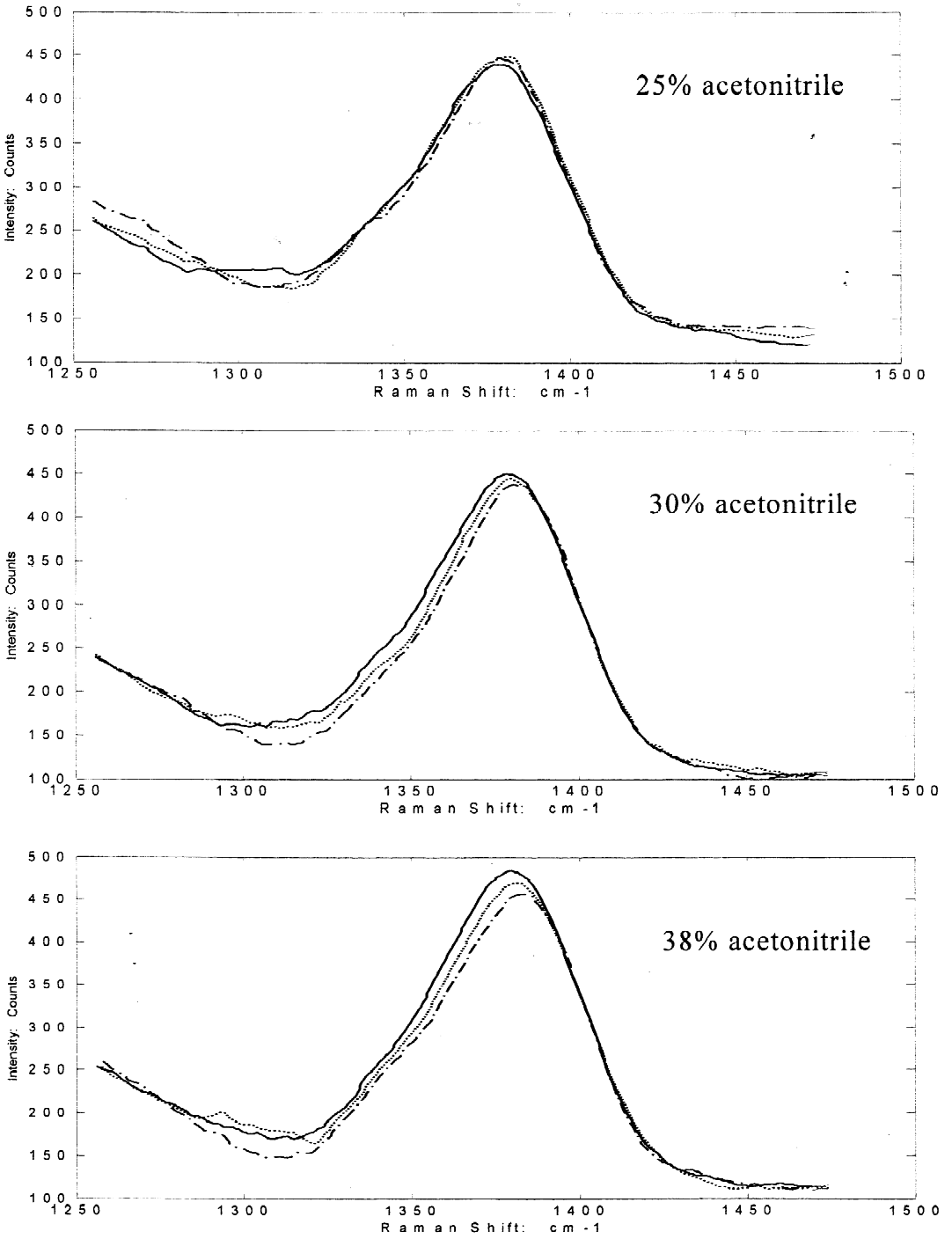

Fig. 6. Raman spectra of the doxorubicin–peptide conjugate as a function of solvent composition and temperature. Solid line = 60 °C, dotted line = 35 °C; dashed and dotted line = 20 °C.

Table 2
Impact of temperature and solvent composition on the ^{1}H NMR spectrum of the doxorubicin–peptide conjugate

	Chemical shift (ppm), 25% (v/v) acetonitrile			Chemical shift (ppm), 38% (v/v) acetonitrile		
	293 K	308 K	333 K	293 K	308 K	333 K
OCH_3	3.77	3.80	3.85	3.84	3.86	3.90
C_2H	7.22	7.27	7.34	7.35	7.38	7.43
C_3H	7.52	7.56	7.64	7.64	7.68	7.72
C_4H	7.37	7.46	7.60	7.59	7.65	7.74
C_7H_2	2.84, 2.55	2.88, 2.64	2.96, 2.77	2.93, 2.68	2.97, 2.75	3.01, 2.84
$C_{10}H$	4.82	4.87	4.95	4.90	4.95	5.02
Leu methyls	0.67, 0.65	0.69, 0.66	0.72, 0.69	0.71, 0.68	0.73, 0.69	0.75, 0.71
Gln-H_2	4.05	4.06	4.10	4.05	4.07	obscured
Chg-h_2	3.86	3.89	3.93	3.88	3.90	3.94

changes in the proximity of the tether to the aromatic ring system of the molecule. The resulting analyte microenvironment is variable and not consistent with the bulk mobile phase environment, which has observable effects on chromatographic performance.

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