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Solution conformation of model peptides with the use of particle beam LC/FT-IR spectrometry and electrospray mass spectrometry

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Abstract

Solution conformations of the peptides angiotensin I (Ang I) and POMC-X (an octapeptide fragment of proopiomelanocortin) were investigated with the use of particle beam liquid chromatography/Fourier transform infrared (LC/FT-IR) spectrometry. Gradient elution high-performance liquid chromatography (HPLC) with mobile phases that contain acetonitrile, 2-propanol, 0.1% heptafluorobutyric acid (HFBA) and 0.1% trifluoroacetic acid (TFA) were used. The conformations of both peptides were studied in 0.9% sodium chloride injection USP, 5% dextrose in water injection USP and sterile water for injection USP. Additional conformational studies over a pH range of 2–10, to a temperature of 75°C and after a storage time of 24 h were investigated. The studies indicated that the two peptides do not behave similarly under identical conditions. It was observed that both Ang I and POMC-X had slightly different conformations in the various parenteral solutions. It was also shown that the conformation of Ang I changed with both pH and temperature while POMC-X was conformationally stable to both temperature and pH. The identity of the peptides and the conformationally sensitive charge-state intensities of the peptides were investigated with electrospray ionization mass spectrometry (ESI/MS). The combination of infrared and mass spectrometric data allowed a thorough estimation of solution effects on the conformations of the model peptides.

Keywords: Angiotensin I; Charge-state intensities; Conformation; Peptides; POMC-X

1. Introduction

Over the past few years, a huge impetus in the field of biotechnology has led to the discovery of a number of bioengineered peptide, polypeptide and protein drugs for the treatment of various diseases [1]. Recently, there has been much concern over the characterization of biopharmaceuticals [2]. One of the major problems associated with these products is their stability in parenteral dosage forms. The complex higher-order structure

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of biopharmaceuticals results in an unpredictable behavior under various conditions and, hence, their chemical and physical properties can be correlated to their efficacy as well as safety. Peptides, polypeptides and proteins have secondary and tertiary structures that pose problems in their purification, storage and administration [3,4]. A loss in the higher-order structure may lead to a change in the pharmacological activity. Peptides and polypeptides are susceptible to peptidases present in the gastro-intestinal tract and hence must be administered intravenously. There is a number of conformational and chemical changes that can occur during the processes of storage and administration which can change the pharmacological activity. Chemical changes are usually monitored with mass spectrometry [5] or amino acid sequencing, but there is no defined methodology to monitor the conformational changes of these biopharmaceuticals in aqueous solution.

High-performance liquid chromatography (HPLC) is a well established and reliable technique for the separation and analysis of peptides and their impurities [6,7]. Classically, ultraviolet detection has been used to detect the analytes and liquid chromatography–mass spectrometry (LC–MS) to confirm their identity. As infrared (IR) spectrometry is very sensitive to secondary structural changes, it has been used successfully in the conformational analysis of peptides and proteins [8]. Delocalized vibrations of the peptide backbone result in amide I, II and III bands, all of which contain secondary structural information [9]. The amide I band (1600–1700 cm^{-1}) is the most conformationally informative and is often utilized for structural studies. Its use in aqueous solution Fourier transform infrared (FT-IR) spectrometry is limited because of the presence of the large absorption band for water in the 1640–1650 cm^{-1} region [10]. In flow-cell LC/FT-IR spectrometry, the low concentration of an analyte makes online IR detection of peptides challenging. An increase in the cell pathlength results in increased absorbance, but the broad and strong IR bands due to water obscure a large portion of the spectrum to the point that sample absorption bands in these regions are undetectable. A solution to this problem is to use an interface that can eliminate the solvent and simultaneously preserve the conformational structure of the peptide or protein. The particle beam LC/FT-IR interface, developed at the University of Georgia, is a low-temperature and -pressure solvent elimination apparatus that restricts the conformational motion of proteins [11–14]. The interface has recently been used to study the conformational states of chromatographically analyzed proteins [15,16]. FT-IR spectrometry has found limited use in the stability studies of peptides because of sample heterogeneity. The degradation of peptides leads to the formation of smaller peptide fragments which can interfere with the IR spectrum of the sample. Also, in formulations that contain these biopharmaceuticals, there are a number of preservatives which can affect the conformation of the peptide. Hence, there is a need to remove these preservatives before the conformational analysis of the peptides is undertaken.

Mass spectrometry (MS) has become a very important tool in the structural characterization of peptides, polypeptides and proteins [5]. It has been used to determine the primary structure of proteins [17] and has frequently been used to detect structural modifications in recombinant proteins [18]. LC–MS is a very powerful technique and has been routinely used for the analysis and characterization of tryptic digests [19]. The ability of electrospray ionization (ESI) to produce intact multiply charged gas phase ions [20,21] from protein molecules in solution has led to its wide use in the conformational analysis of proteins [22]. Amide hydrogen/deuterium exchange rate measurement by ESI/MS has been used as an analytical technique to probe protein secondary structure. This technique has been developed over the last few years but is limited by the fact that only approximate estimates of α-helical, β-sheet and random coil structures can be obtained [23,24].

The objective of this study was to investigate the use of particle beam (PB) LC/FT-IR spectrometry as an analytical technique to study the conformational changes of peptides in solution. Angiotensin I (Ang I), a decapeptide, and POMC-X, an octapeptide fragment of proopiomelanocortin, were chosen as model peptides. The

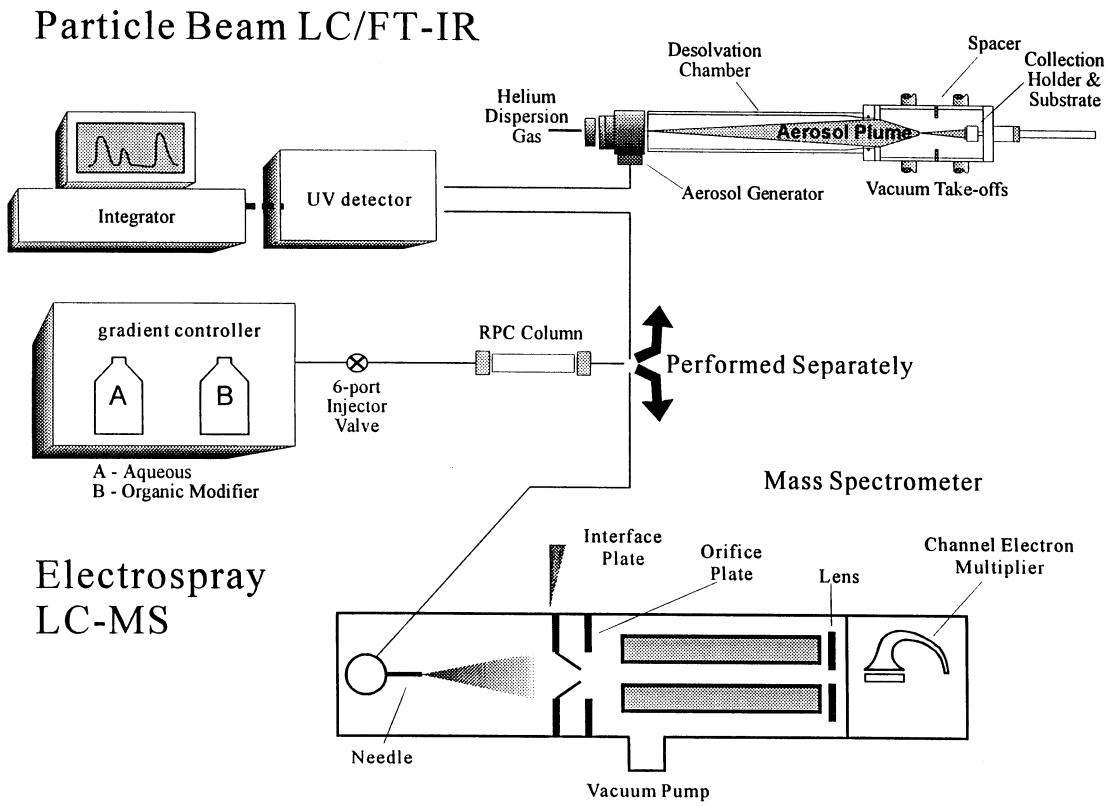

Fig. 1. Schematic of the particle beam LC/FT-IR spectrometer and the electrospray LC-MS.

peptides were reconstituted in various parenteral mixtures such as 0.9% sodium chloride injection USP, 5% dextrose in water injection USP (D5W) and sterile water for injection USP, and the solution effect on the conformation of the peptides was investigated. The effects of pH, temperature and time on the conformation of the model peptides in aqueous media were studied to assess their structural stability. Electrospray MS was used as an additional technique to particle beam LC/FT-IR spectrometry. Studies have indicated that particle beam LC/FT-IR spectrometry can be used effectively to identify conformational changes in these model peptides.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of tandem Varian model 2510 HPLC pumps (Walnut Creek, CA) connected to a Varian static mixer. The mixer was attached to a Rheodyne model 7125 manual injector (Cotati, CA) equipped with a 100 μl loop. A Vydac (Hesperia, CA) octadecylsilane analytical column (4.6 × 250 mm, 5 μm, 300 Å) was used to separate the peptides from other components. A Micromeritics (Norcross, GA) model 787 variable wavelength UV-Vis detector was used to detect

chamber, a single momentum separator and a 5 the analytes. The output was recorded using a Spectra Physics model 4290 Integrator (San Jose, CA). The UV detector was connected to the particle beam interface (Fig. 1). The particle beam interface was equipped with a 22 cm desolvation cm nozzle to substrate distance. The column effluent was converted into an aerosol with 25 μm silica tubing (Polymicro Technologies, Phoenix, AZ). The column was thermostatted at ambient temperature (23°C) using a column heater (Fiatron Systems, Milwaukee, WI). Desolvated peptide deposits were collected onto calcium fluoride disks (25 × 2 mm, International Crystal Laboratories, Garfield, NJ) and interrogated off-line using a Spectra Tech IR-Plan™ infrared microscope (Shelton, CT) interfaced to a Perkin–Elmer 1725X FT-IR spectrometer (Norwalk, CT). All spectra were obtained from 1000 scans at 8 cm^{-1} resolution. All spectral manipulations, including second derivative and deconvolution, were performed with GRAMS 386™ software (Galactic Industries, Salem, NH). The mass spectral data were obtained with a VG Quattro II triple quadrupole mass spectrometer (Micromass, Beverly, MA). Solution state spectra of the model peptides were obtained using an attenuated total reflectance (ATR) micro CIRCLE cell accessory (Spectra-Tech, Shelton, CT) in a FTS-60A/896 infrared spectrometer (BioRad Laboratories, Digilab division, Cambridge, MA). Diffuse reflectance spectra were obtained using a DR accessory (Spectra Tech, Shelton, CT) in an IR spectrometer (Perkin–Elmer, Norwalk, CT).

2.2. Reagents and chemicals

Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) was obtained from Sigma Chemical Company (St. Louis, MO). POMC-X (Trp-Cys-Leu-Asp-Ser-Ser-Gln-Cys) was synthesized at the molecular genetics facility in the University of Georgia (Athens, GA). The purity of POMC-X was confirmed by HPLC to be 96%. Trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were also obtained from Sigma. Water was purified by a cartridge system (Continental Water Systems, Roswell, GA). Acetonitrile (Fisher Chemical, Pittsburgh, PA) was HPLC grade and all other reagents used in the assay were Baker Analyzed reagents.

2.3. Preparation of solutions

Individual solutions (0.5 mg ml^{-1}) of Ang I and POMC-X were prepared by weighing 0.5 mg of each peptide in individual 1 ml volumetric flasks and 0.9% sodium chloride injection USP, D5W USP, sterile water for injection USP and 5% sodium chloride solution were added to volume. Solutions of the peptides in the different parenteral carriers were stored at ambient temperature (23 ± 2°C) and aliquots were removed and assayed at 0 and 24 h.

Ammonium acetate solutions (10 mM) of pH 2.25, 4.0, 6.0, 8.0 and 10.0 were prepared for the pH studies. Either glacial acetic acid or ammonium hydroxide was used to adjust the pH. Solutions of the model peptides in each acetate buffer were prepared by weighing of the appropriate amounts of the peptides into separate 1 ml volumetric flasks and the corresponding buffer added to volume. In the case of temperature studies, individual solutions of Ang I and POMC-X were prepared in water and divided into two parts. One part was stored at ambient temperature (23 ± 2°C) and the second part was heated at 75°C in a water bath.

2.4. Chromatographic conditions

(**I**) The gradient elution chromatographic conditions used for the separation of Ang I were as follows. Column: Vydac C-18 (250 × 4.6 mm, 5 μm, 300 Å); mobile phase:

A: 90:10 (v/v) 0.1% HFBA–acetonitrile
B: 80:20 (v/v) acetonitrile–0.1% HFBA

Time (min)	% A	% B
0	70	30
40	50	50

Flow rate, 0.4 ml min^{-1}; injection volume, 100 μl; detection wavelength, 220 nm.

(**II**) The gradient elution chromatographic conditions for POMC-X were as follows. Column: Vydac C-18 (250 × 4.6 mm, 5 μm, 300 Å); mobile phase:

A: 90:7:3 (v/v/v) 0.1% TFA–acetonitrile–2-propanol
B: 10:70:20 (v/v/v) 0.1% TFA–acetonitrile–2-propanol

Time (min)	% A	% B
0	85	15
60	45	55

Flow rate, 0.4 ml min^{-1}; injection volume, 100 μl; detection wavelength, 220 nm.

2.5. Evaporated films, ATR and solution state spectra

Ang I and POMC-X (0.5 mg each) were weighed into separate 1 ml volumetric flasks and deionized water was added to volume. Solid films were prepared by evaporation of each solution under vacuum onto a calcium fluoride disk. The resultant deposit was examined under the IR microscope to obtain a spectrum of each peptide. The solution state spectra of the peptides were obtained from 2–3% (w/v) solutions of the peptides with the use of an attenuated total reflectance (ATR) CIRCLE cell accessory. Spectral subtraction was used to eliminate bands due to water. Diffuse reflectance experiments (DR) were carried out with small quantities of the crystalline peptides mixed individually with dried potassium bromide.

2.6. Particle beam deposit collection

Deposits of the model peptides were collected with the use of the particle beam LC/FT-IR spectrometer shown in Fig. 1. The deposits were collected onto a calcium fluoride disk and investigated with the use of an IR microscope as previously described by Turula and de Haseth [11,12]. Silica tubing (25 μm i.d.) was used to produce an aerosol and connect the UV detector to the particle beam interface. Care was taken to ensure that the HPLC column back pressure did not exceed 4000 psi. The distance of the calcium fluoride disk from the momentum separator in the interface was optimized at 5 mm to obtain uniform deposits of approximately 50–100 μm in diameter.

2.7. Mass spectral collection

The masses of the peptides Ang I and POMC-X were confirmed by LC–MS. A megaflow electrospray probe was used and the peptides were analyzed under the chromatographic conditions described previously. The peptides were collected and reconstituted in ammonium acetate buffers of various pH values. Ammonium acetate buffers of various pH values were also used as the mobile phase carriers and the peptides were flow injected (KD Scientific model 200 syringe pump) into the system. The flow rate was set at 10 μl min^{-1} for both peptides. The needle and cone voltages for the analysis of Ang I were 3.30 kV and 50 V, respectively, and 3.11 kV and 69 V, respectively, for POMC-X. The data were obtained in triplicate and analyzed further, as detailed below.

3. Results and discussion

Angiotensin I (Ang I) and POMC-X (B) eluted at 24.2 and 13.4 min, respectively, under the HPLC gradient elution conditions described above. Typical chromatograms of the two peptides are shown in Fig. 2(A) and (B). Ang I (B) was well resolved from its impurities (A). They (Ang I and POMC-X) were individually collected with the particle beam interface onto a calcium fluoride window and subjected to infrared analysis. The particle beam technique uses an aerosol to eliminate the mobile phase by low-temperature evaporation from the analyte molecules. In normal operation, the peptide solution is pumped through a small orifice and exits as a liquid jet which is nebulized with a stream of helium [11]. Using 25 μm fused silica capillary, the optimum flow rate to obtain a good deposit with minimum back pressures is usually 0.3–0.5 ml min^{-1}. The flow rate used in these experiments was found to be 0.4 ml min^{-1} since higher flow rates resulted in back pressures greater than 5000 psi. The nebulized sample was directed into a low-pressure chamber where rapid desolvation occurred. The

peptide was then deposited onto a calcium fluoride window, which was removed from the interface and the deposited peptide analyzed off-line using FT-IR microscopy. The size and nature of the peptide deposits were optimized by varying the distance between the window and the skimmer. Experiments performed in this laboratory with distances of 4–6 mm indicated that a distance of 5 mm gave a uniform and compact deposit of the peptide. The desolvation process is endothermic and occurs in a few milliseconds, such that the low-energy solution particles are trapped as aerosol particles. Thus, rapid isolation of the peptide from solution produced a dried deposit, the IR spectrum of which was highly representative of the peptide's solution state structure. The conformational analysis of β-lactoglobulin and lysozyme with the particle beam technique has demonstrated that the various processes such as nebulization, desolvation and substrate collection do not affect protein structural integrity [11]. The preservation of biological activity during particle beam LC/FT-IR spectrometry has also been confirmed with a series of spectrophotometric biological assays reported by Turula and de Haseth [11]. In addition, changes in the solution charge-state distribution observed with changes in solvent composition, pH and temperature have led to the hypothesis that transport of proteins from the solution phase to the gas phase via the electrospray interface maintains higher-order protein secondary structure [23]. Since it has been shown that particle beam and electrospray interfaces produce spectra that are indicative of solution state secondary structure of proteins, they were used in this laboratory to study the conformation of the model peptides Ang I and POMC-X in solution.

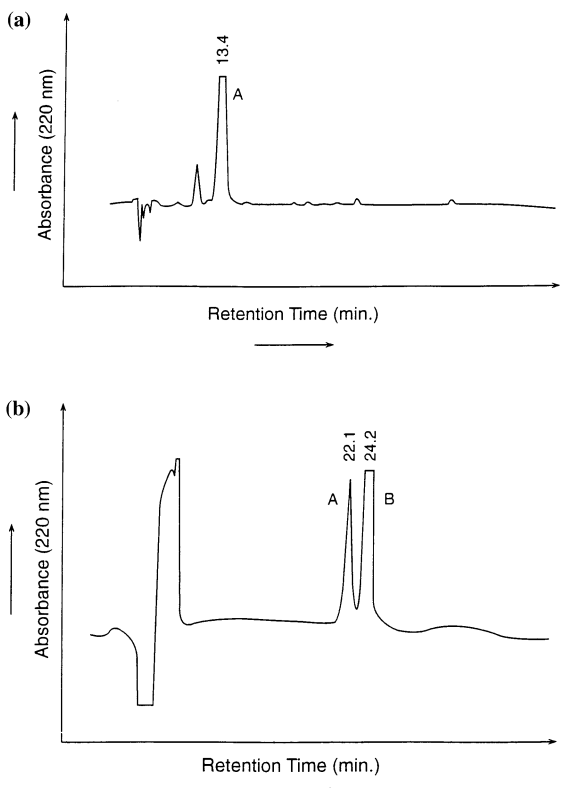

Fig. 2. (a) Typical chromatogram of POMC-X (A) in mobile phase. For chromatographic conditions, see Section 2. (b) Typical chromatogram of Ang I (B) and other unknown impurities (A) in 0.9% sodium chloride injection USP. For chromatographic conditions, see Section 2.

Evaporated films of Ang I were prepared by placing a drop of a concentrated peptide solution (in water) onto a calcium fluoride disk and evaporating under vacuum. The evaporated film when examined with IR microscopy showed the predominance of a β-sheet conformation. This was highlighted by the presence of a strong amide I band at 1639 cm^{-1} (Fig. 3(A)). Mild deconvolution and second derivative spectroscopy (Fig. 3(B)) were further used to confirm the presence and position of these bands. A comparison of the evaporated film, solution state and particle beam spectra (Fig. 3(A)) indicated that similar bands existed in all spectra of Ang I. A band at 1662 cm^{-1} was found in all three spectra but was slightly more prevalent in the PB spectra and is indicative of some disordered conformation in Ang I. The presence of both the disordered band as well as the β-sheet band was indicative of a population equilibrium. It was inferred that depending on the solution state conditions, a certain percentage of Ang I molecules had a disordered band conformation while the rest had a β-sheet conformation. The high degree of similarity in the IR spectra indicated that particle beam LC/FT-IR spectrometry did not alter the conformation of the peptide and can, therefore, be used for the

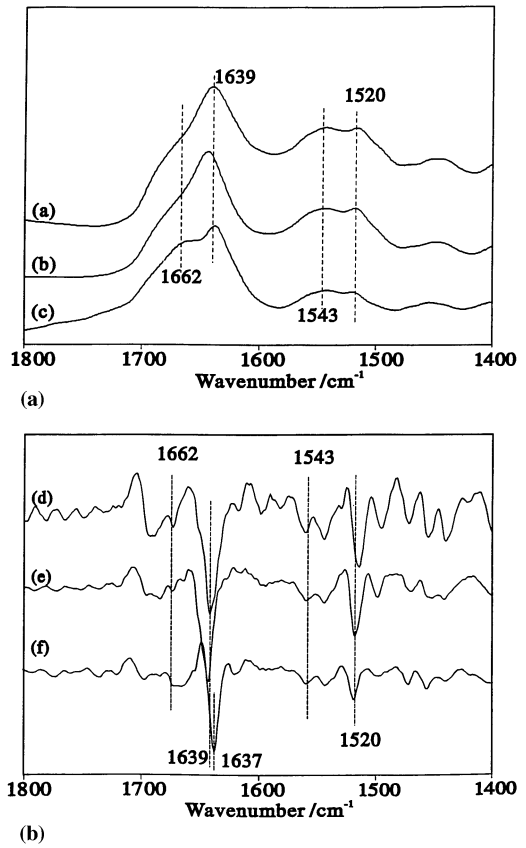

Fig. 3. A: Infrared spectra of angiotensin I from (a) crystalline deposit, (b) evaporated film deposit and (c) particle beam deposit. B: Second derivative spectra of Ang I obtained from (d) crystalline deposit, (e) evaporated film and (f) particle beam deposit.

conformational analysis of Ang I in solution. The amide II bands at 1520 and 1545 cm^{-1} were also indicative of a β-sheet conformation. Ang I was also investigated by diffuse reflectance spectroscopy and the presence of all the bands seen in the evaporated film, crystalline deposit and the particle beam spectra was further confirmed.

The evaporated film of POMC-X displayed results identical to those seen previously with Ang I (spectrum not illustrated). There was the predominance of a β-sheet band at 1640 cm^{-1} in all the spectra. The presence of another strong band at 1662 cm^{-1} was indicative of the presence of disordered conformation. There was a weak band at 1652 cm^{-1} which represented a small percentage of α-helical character. POMC-X was investigated by diffuse reflectance spectroscopy and the presence of all the bands seen in the evaporated film, crystalline deposit and the particle beam spectra was further confirmed. Molecular modeling studies further confirmed all the observations seen in the above POMC-X studies (A.G. Cox, T.G. Venkateshwaran, E.W. Taylor, J.T. Stewart, unpublished results, University of Georgia, Athens, GA, USA, 1996).

Ang I and POMC-X were then reconstituted in 0.9% sodium chloride injection USP, D5W USP and sterile water for injection USP. The conformation of the model peptides was examined over a 24 h period (Figs. 4 and 5). HPLC effectively separated the peptides from any impurities formed over this period of time and PB LC/FT-IR spectrometry was used to monitor the conformational changes of each peptide. When peptide deposits collected from the parenteral solutions were examined, it was observed that the IR spectra of Ang I deposits from sterile water for injection (Fig. 4(d)) and 0.9% sodium chloride injection (Fig. 4(c)) were similar. The spectra contained strong peaks at 1636–1640 cm^{-1} which were representative of a β-sheet conformation and a band at 1664 cm^{-1} that represented disordered conformation. The IR spectrum of Ang I deposits obtained from D5W (Fig. 4(b)) was slightly different in that the β-sheet wavenumber

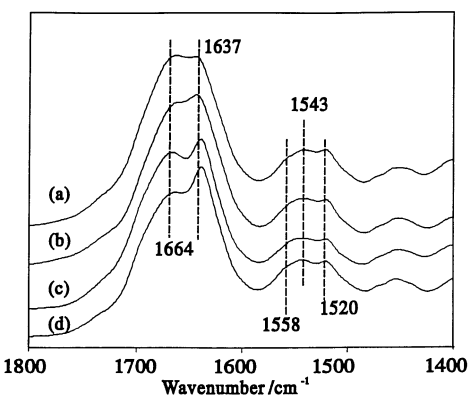

Fig. 4. Infrared spectra of Ang I: particle beam deposits collected from (a) 5% sodium chloride injection USP, (b) 5% dextrose USP, (c) 0.9% sodium chloride USP and (d) sterile water for injection USP.

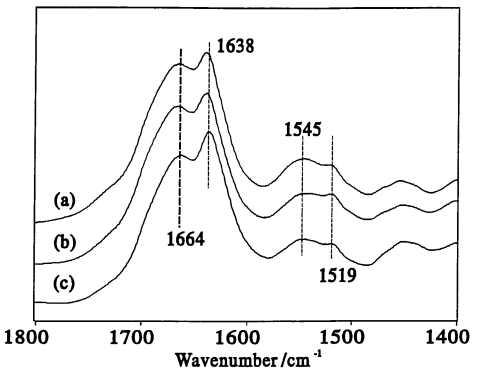

Fig. 5. Infrared spectra of Ang I: particle beam deposits collected after 24 h from (a) 5% dextrose USP, (b) 0.9% sodium chloride solution USP and (c) sterile water for injection USP.

value was shifted to 1642–1643 cm^{-1}. This wavenumber shift might indicate a very slight change in the conformation of Ang I in D5W. In addition, the relative intensity of the disordered band at 1662 cm^{-1} was greater in D5W than in either sterile water or 0.9% sodium chloride injection. This difference in relative intensity of the disordered band to that of the β-sheet band was indicative of a change in the population equilibrium of Ang I. In order to observe whether a higher concentration of salt in solution would have a similar effect on Ang I population distribution, a 5% solution of sodium chloride in water was prepared in our laboratory and similar studies were performed. The spectrum of Ang I obtained from the 5% sodium chloride solution (Fig. 4(a)) was identical both in intensity and in the position of bands to that obtained with D5W. Spectra of Ang I obtained from different solutions were stored for 24 h (Fig. 5) showed slight differences in band intensities to the spectra collected at 0 h. The IR spectra of Ang I deposits collected from D5W (Fig. 5(a)) indicated that the relative intensity of the β-sheet was greater after 24 h which indicated that the molecule had undergone a change in the population equilibrium over that period of time. This was also observed in the spectra of Ang I deposits from the 5% sodium chloride solution (spectrum not illustrated). Spectra of Ang I collected from sterile water (Fig. 5(c)) and 0.9% sodium chloride injection (Fig. 5(b)) after storage for 24 h at ambient temperature (23 ± 2°C) also showed no shifts in the amide I bands, but there was a decrease in the relative intensity of the β-sheet bands compared to the disordered bands, indicating a slight change in the population equilibrium in these solutions over time. The IR spectra of Ang I obtained from the PB deposits collected from all three solutions were identical to each other. Whether these changes in conformation lead to a change in biological activity or degradation of the POMC-X, Ang I or both is unknown.

Particle beam spectra of POMC-X from sterile water, 0.9% sodium chloride injection and D5W are presented in Fig. 6. There was a strong band at 1638 cm^{-1} in all spectra, which indicated the presence of a β-sheet conformation. The presence of a strong band at 1663 cm^{-1} indicated the presence of disordered conformation in the molecule. Mild deconvolution and second derivative spectroscopy were used to identify the position of certain bands. A band at 1695 cm^{-1} further confirmed the presence of a β-sheet in the molecule. The relative intensity of the disordered conformation band compared to the β-sheet band was different in the deposits of POMC-X collected from the various parenteral solutions. This is once again indicative of a population equilibrium between the β-sheet and the disordered conformation. The band intensity ratio of disor-

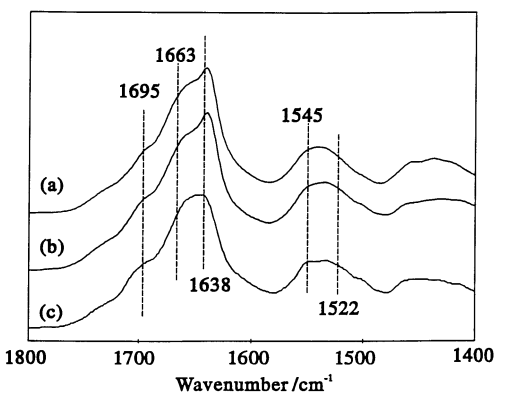

Fig. 6. Infrared spectra of POMC-X: particle beam deposits collected from (a) 5% dextrose USP, (b) 0.9% sodium chloride injection USP, and (c) sterile water for injection USP.

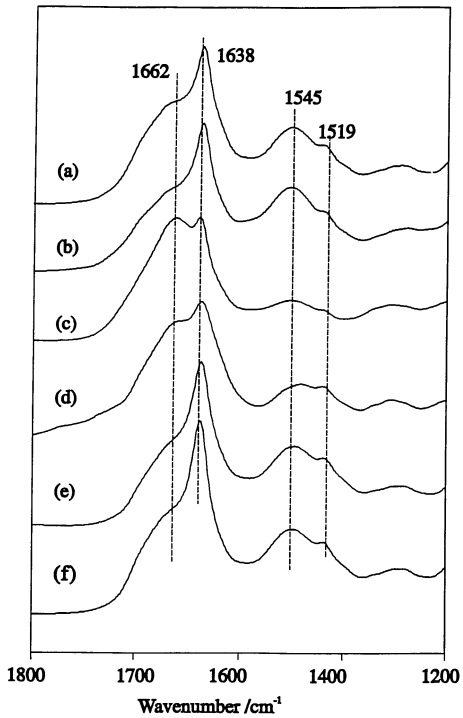

Fig. 7. Ang I: infrared spectra of particle beam deposits from (a) pH 2.5, (b) pH 4.0, (c) pH 6.0, (d) pH 7.0, (e) pH 8.0 and (f) pH 10.0.

dered conformation to β-sheet was greater in sterile water than 0.9% sodium chloride injection or D5W. Particle beam deposits collected from sterile water for injection after a 24 h storage period (ambient temperature 23 ± 2°C) when compared to spectra obtained at 0 h, indicated practically no change in the conformation of the molecule over time.

Particle beam studies with different buffers were performed previously in this laboratory. It was determined that ammonium acetate was the most compatible buffer salt due to its volatility [25]. Ammonium acetate buffers of various pH values were used to reconstitute the peptides and PB deposits were collected. The IR bands of Ang I indicated the presence of a β-sheet conformation under all conditions with a band at 1638 cm^{-1} (Fig. 7). Nonetheless, the intensity of the disordered conformation (1660–1670 cm^{-1}) band relative to the β-sheet band varied slightly with a change in pH which indicates a shift in population equilibrium with a change in solution state environment. The population equilibrium shifted towards a disordered form as the pH increased and reached a maximum at pH 6.0. The disordered band was prominent in water (pH 7.0). But as the pH increased further (7–10), the population equilibrium shifted more towards a β-sheet conformation. The data was interpreted under the assumption that the population equilibrium at a pH of 7.0 (water) was representative of the native conformation of Ang I. As the pH increased or decreased, there was a change in the population equilibrium indicating that there was a change in the conformation of the molecule with pH.

Peptides and proteins are very susceptible to denaturation at extremes of pH. Denaturation leads to unfolding of the biomolecules which leads to a change in the ESI charge state intensities. As the peptides and proteins unfold, the number of exposed amino acids increase, thereby increasing the number of ionizable sites in the molecule. This leads to a shift in the charge state intensities towards lower m/z values. Ang I, a decapeptide, has only three charge states. Hence, a shift in the charge state intensity envelope is not seen very clearly. In our studies, the intensity ratio of the singly to triply charged ions has been used as a tool to analyze the change in conformation with pH. The mass spectral data obtained for Ang I under the various pH conditions shown in Table 1 indicated a substantial difference in the ratio of the singly charged to the triply charged state as a function of pH. It was observed that this ratio increased as the pH increased from 2.0 to 6.0 and then decreased as the pH increased from 6.0 to

Table 1
Ratio of charge-state intensities for Ang I under various conditions

Angiotensin I conditions	C[a]
pH 2.0	2.07
pH 4.0	4.72
pH 6.0	11.11
pH 8.0	7.60
pH 10.00	5.78

[a] Represents charge-state intensity at m/z 1296 (1^+) and m/z 433.3 (3^+).

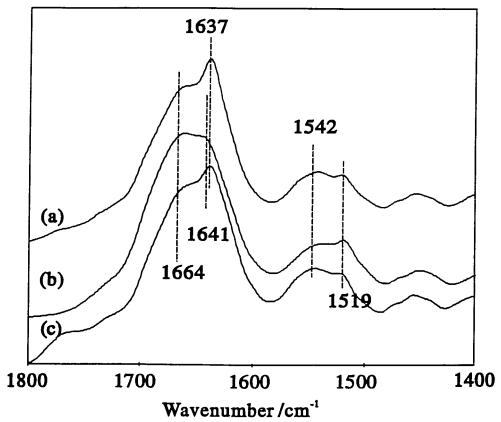

Fig. 8. Ang I: infrared spectra of particle beam deposits collected at (a) room temperature, (b) 75°C, (c) exposed to 75°C and left at ambient temperature for 24 h.

10.0. This indicated that there was a change in the charge state intensities of Ang I with pH that is consistent with the IR data.

The deposits of POMC-X collected at the same pH values showed no significant variation in the IR spectra (spectra not illustrated). Therefore, POMC-X was thought to be conformationally stable in the pH range examined. The mass spectral data obtained for POMC-X did not have a change in the intensities of the singly and doubly charged species. The intensity of the doubly charged species was extremely low and this intensity could not be increased with a change in the needle or cone voltage. It was observed, however, that the increase in pH led to a change in the intensity of reduced to oxidized peaks. The intensity of the reduced POMC-X ($m/z = 940$) was greater at low pH, but as the pH increased, the intensity ratio of the oxidized peak ($m/z = 938$) and the reduced peak ($m/z = 940$) was not consistent. This indicated that the molecule was not in one form and was consistently changing from the oxidized to reduced form and vice versa. This was not seen as a clear difference in the IR spectra. A possible explanation for this could be that the process was too dynamic to cause a significant change in the IR spectra of a small molecule such as POMC-X.

The effect of temperature on the conformation of the peptides was also examined (Fig. 8). When Ang I was heated at 75°C for 30 min, the intensity of the disordered conformation band relative to the β-sheet band increased considerably which indicates a change in the conformation of the peptide at the higher temperature (Fig. 8(b)). When a deposit of Ang I solution heated at 75°C was allowed to sit at ambient temperature (23 ± 2°C) for 24 h (Fig. 8(c)), it was observed that the relative intensity of the disordered band was less than that of the deposit collected immediately after heating, indicating that the peptide may have a tendency to return to the original equilibrium distribution.

4. Conclusions

Particle beam LC/FT-IR spectrometry has been shown to be a useful tool for the investigation of conformational changes in small peptides such as Ang I and POMC-X. Ang I was believed to exist in an equilibrium between the disordered and the β-sheet conformation. A change in the solution state conditions led to a change in this equilibrium which indicates a change in the population conformation. PB deposits of Ang I obtained from D5W showed a slight difference in population equilibria at 0 h which was not observed at 24 h and indicates a change in conformation over time. These changes were not observed with POMC-X. An increase in pH from 2 to 7 resulted in the predominance of a disordered structure band for Ang I, but at pH greater than 7, there was an increase in β-sheet intensity and indicates a shift in the population equilibrium of Ang I over time. A similar change was observed for Ang I with ESI/MS where the intensity ratio of singly to triply charged ions initially increased as the pH increased from 2 to 7, and then decreased at pH greater than 7. With POMC-X, there were minimal conformational changes observed at all pH levels. Temperature studies with Ang I indicated a change in population equilibrium characterized by the predominance of a disordered conformation at higher temperatures. Presently, the effects of various parenteral formulations on the conformation of peptides are being investigated. Additional MS experiments are underway to study conforma-

tional changes and to investigate any correlation between MS and IR data.

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References

[1] C.J. Wordell, Am. Drug 202 (1990) 18–26.
[2] W.S. Hancock, B. Willis, Am. Lab. June (1996) 31–34.
[3] M.C. Manning, K. Patel, R.T. Borchardt, Pharm. Res. 6 (11) (1989) 903–918.
[4] T. Chen, Drug Dev. Ind. Pharm. 18 (11/12) (1992) 1311–1354.
[5] J.S. Andersen, B. Svensson, P. Roepstoff, Nature Biotechnol. 14 (1996) 449–457.
[6] M.I. Aguilar, M.T.W. Hearn, in: M.T.W. Hearn (Ed.), HPLC of Proteins, Peptides and Polynucleotides, VCH, New York, 1991, pp. 247–275.
[7] P. Oroszlan, R. Blanco, X.M. Xu, D. Yarmush, B.L. Karger, J. Chromatogr. 500 (1990) 481–502.
[8] A. Elliot, E.J. Ambrose, Nature 165 (1950) 921–922.
[9] H. Susi, in: S.N. Timasheff, G.D. Fasman (Eds.), Structure and Stability of Biological Molecules, Marcel Dekker, New York, 1969, pp. 575–633.
[10] S.N. Timasheff, H. Susi, L. Stevens, J. Biol. Chem. 242 (1967) 5467–5473.
[11] V.E. Turula, J.A. de Haseth, Appl. Spectrosc. 48 (1994) 1255–1264.
[12] J.A. de Haseth, V.E. Turula, Mikrochim. Acta (1997), in press.
[13] R.T. Bishop, V.E. Turula, J.A. de Haseth, R. Ricker, Techniques in Protein Chemistry VIII, Elsevier, Amsterdam (1997), in press.
[14] V.E. Turula, R.T. Bishop, R. Ricker, J.A. de Haseth, J. Chromatogr. A 763 (1997) 91–103.
[15] V.E. Turula, J.A. de Haseth, Anal. Chem. 68 (1996) 629–638.
[16] R.T. Bishop, V.E. Turula, J.A. de Haseth, Anal. Chem. 68 (1996) 4006–4014.
[17] J.A. Loo, C.G. Edmonds, R.D. Smith, Science 248 (1990) 201–204.
[18] K.L. Biemann, H.A. Scoble, Science 237 (1987) 992–998.
[19] E.C. Huang, J.D. Henion, J. Am. Soc. Mass Spectrom. 1 (1990) 158–165.
[20] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64–71.
[21] R.D. Smith, J.A. Loo, C.G. Edmonds, C.J. Barinaga, H.R. Udseth, Anal. Chem. 62 (1990) 882–899.
[22] S.K. Chowdhury, V. Katta, B.T. Chait, J. Am. Chem. Soc. 112 (1990) 9012–9013.
[23] C.L. Stevenson, R.J. Anderegg, R.T. Borchardt, J. Am. Soc. Mass Spectrom. 4 (1993) 646–651.
[24] Y. Liu, D.L. Smith, J. Am. Soc. Mass Spectrom. 5 (1994) 19–28.
[25] R.B. Robertson, J.A. de Haseth, R.F. Browner, Appl. Spectrosc. 44 (1) (1990) 8–13.

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