Monitoring Protein Modifications of the Biogeneric Protein, Interferon-α, via Reversed Phase Chromatography

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Abstract
Physical and chemical degradation of therapeutic proteins is a critical problem that can occur during production, purification, and storage. Such modifications can affect protein immunogenicity leading to serious consequences if the protein is being used as a therapeutic. Degradation processes such as unfolding, mis-folding, aggregation, methionine oxidation, and asparagine/glutamine deamidation were studied; analytical methods to detect such modifications by reversed phase chromatography and peptide mapping were developed. Analytical methods for Human Interferon-α, a protein being manufactured as a biogeneric, were optimized using different Jupiter® 300 & Proteo reversed phase HPLC columns. The most recent addition to the Jupiter portfolio, Jupiter 300 3 µm C18 provided the best separation for intact analysis of Interferon-α post-translational modifications as well as delivered the best peptide map profile.

Introduction
Interferon represents a family of proteins characterized by their non-specific antiviral and antiproliferative activity. This glycoprotein belongs to the cytokines class of proteins and has been useful as an antiviral and anticancer drug[1, 2]. In addition to a direct antiviral effect, it also exhibits potent immunostimulatory activity[3]. The use of interferon as a therapeutic drug dates back to the 1970's and is classified into three types: α-Interferon, β-Interferon, and γ-Interferon, based on serological and structural relations. Interferon-α (IFN-α) is most widely used for therapeutic purposes. Interferons are also known to affect every component of the immune system and long-term IFN-α therapy has induced systemic autoimmunity[4]. A critical area in the research of therapeutic proteins is the study of their stability and post-translational modifications (PTMs). Common protein modifications include variable glycosylation, mis-folding and aggregation, oxidation of methionine, deamidation of asparagine and glutamine residues, and proteolysis. These modifications not only pose challenges for accurate and consistent bioprocessing, but also may have consequences for the patient in that incorrect modifications or aggregation may lead to an immune response to the protein therapeutic.

Patents for many of the first generation protein therapeutics have recently expired opening the possibility of manufacturing proteins, like Interferon-α, in a biogeneric mode. Such new materials will require analytical methods to demonstrate equivalence to first generation products as well as monitor the quality of the protein. This study provides examples of chromatographic analysis for stability studies of human Interferon-α (HuIFN-α) protein. We compared different reversed phase media and different size particles to separate and to demonstrate most common modifications during production, storage, and stability studies of therapeutic proteins.

Materials and Methods
Recombinant human protein Interferon-α (CHO expression source) was obtained from Abcam inc. (Cambridge, MA, USA)). Sequencing Grade Trypsin was purchased from Roche Applied Science (Indianapolis, IA, USA). Solvents were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Analyses were performed using a HP 1100 LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a DAD detector. Various HPLC columns were used for evaluations including Jupiter 300 5 µm C18, Jupiter 300 3 µm C18, Jupiter 300 5 µm C4, Jupiter Proteo 4 µm, all 150 x 2.0 mm (Phenomenex, Torrance, CA, USA). Aqueous mobile phase used was 0.1 % TFA and 2 % ACN in water and a gradient of organic mobile phase, 90 % acetonitrile/0.085 % TFA in water was used. Tryptic digests of Interferon-α were analyzed with a gradient from 1 to 50 % of organic mobile phase in 35 minutes followed by a 50 to 80 % gradient in 5 minutes. For analysis of intact Interferon-α (unmodified and PTM modified protein) the gradient used was from 20 to 80 % organic in 10 minutes followed by a 5 minute flush with 90 % organic. Flow rate for all analyses was 0.3 mL/min; protein elution was monitored at 220 nm. For intact protein analysis 2 µg was injected; for protein digests 8 µg of the digest was injected.

Oxidation of intact Interferon-α was performed by treating with 3 % hydrogen peroxide and 50 mM sodium citrate pH 4.4 at 45 °C for 120 minutes. *Deamidation* was performed in 50 mM Sodium citrate/25 mM Tris pH 9.0 at 45 °C overnight. *Unfolding* was performed by treating the intact protein with 3 mM dithiothreitol (DTT) at 55 °C for 30 minutes. *Tryptic digestion* was performed in 30 mM ammonium bicarbonate pH 8.0 for 5 hrs at 37 °C; ratio of enzyme to substrate was 1/20 w/w. For some protein digests an aliquot was *reduced* with 3 mM DTT at 45 °C for 30 minutes. Digestion and reduction reactions were quenched by lowering the pH with 20 % TFA (pH~3).

Results and Discussion
Regulatory agencies require several different tests for lot release and stability testing of therapeutic proteins. Reversed phase chromatography is usually used for two of these tests: intact protein analysis and peptide mapping. Intact protein analysis using reversed phase chromatography is often useful for identifying unfolded and mis-folded proteins; sometimes such techniques can also visualize other post-translational modifications. However, peptide mapping is the best technique for identifying post-translational modifications and almost always uses reversed phase chromatography for the separation of the released peptides. In this technical note, both techniques were shown for Interferon-α using the Jupiter media as an example of how such materials can be used for biogeneric protein applications.

APPLICATIONS

Peptide Mapping

Peptide mapping, using different proteases, is a good analytical method to screen PTMs of therapeutic proteins. As an example, the proteolytic analyses of protein with trypsin on different Jupiter phases were demonstrated in this technical note. Well-studied human Interferon-α, a 19.2 kDa protein containing two disulfide bonds (cys1–cys98; cys29–cys138) was tryptic digested and then reduced with DTT. Both samples were well separated on Jupiter® 300 3 µm C18 column. **Figure 1** shows peptides retention time shifting due to disulfide bond breakage after reduction reaction. Such shifts are clearly observed in the peak distribution at different retention times of the trypsin digested Interferon-α (**Figure 1**: Blue chromatogram) and Interferon-α digested and reduced (**Figure 1**: Red chromatogram). Such shifts are indicative of a disulfide containing peptide and are easily verified by mass spectrometry.

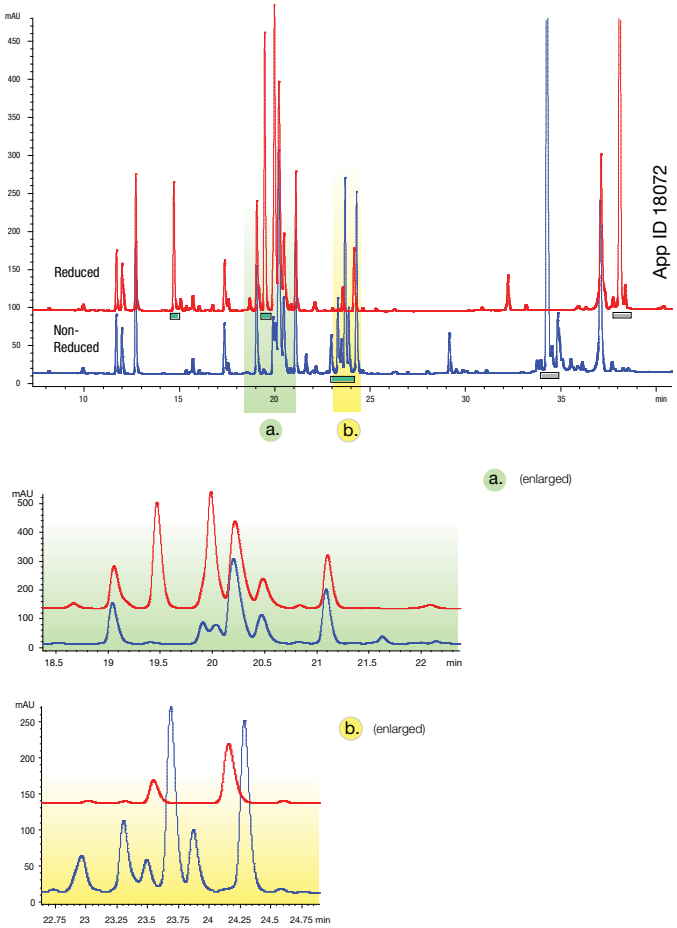

Figure 1.
Separation of the tryptic digest of Human Interferon-α (chromatogram in blue) and Human Interferon-α digested with trypsin and reduced with DTT (chromatogram in red) on Jupiter 300 3 µm C18 150 x 2.0 mm column. Disulfide bond cleavage after DTT reduction of HuIFN-α is easily observed when comparing the two chromatograms. Zoom in views of different sections of the chromatogram better show the differences between the reduced and non-reduced peptide maps.

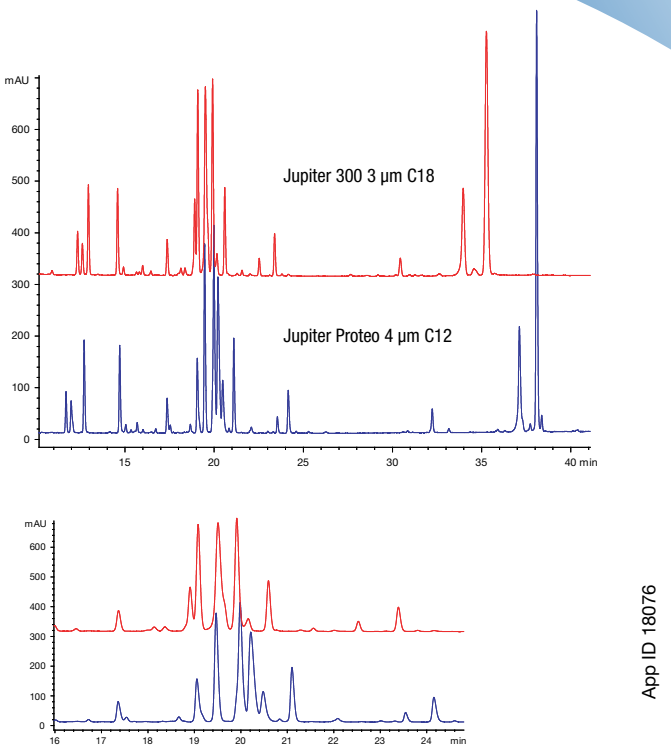

Figure 2.
Peptide maps of Hu IFN-α run on the Jupiter 300 3 µm C18 (Red) and the Jupiter Proteo 4 µm C12 (Blue). Close in zoom between 16-25 minutes indicates that the Jupiter 300 3 µm C18 provides slightly better resolution of the closely eluting peptides.

To define better selectivity and resolving power for peptide mapping, several reversed phase columns were selected. Considering different silanol activity for different bonded phase media and their sizes, the C12 and C18 with 3 and 4 µm particle size, and 90 and 300 Å pore size media were evaluated. **Figure 2** shows reversed phase separations of digested and reduced HuIFN-α on different medias: Jupiter Proteo 4 µm C12 and Jupiter 300 3 µm C18. While both Jupiter columns give excellent separation of all the digested peaks, the inserted zoom view suggests that for this particular peptide separation the Jupiter 300 3µm C18 delivers slightly better resolution of closely eluting peptides. The increased surface area and bonding density of the Jupiter Proteo media will often provide better separation power than 300 Å medias; however, in this example the Jupiter 300 3 µm C18 outperforms the Proteo indicating that both media should be evaluated when developing a peptide mapping application.

Intact Protein Analysis

Besides peptide mapping, reversed phase chromatography is often used in monitoring the folded state of a protein. Unlike other separation techniques, reversed phase can often visualize differences between intact and unfolded/ mis-folded protein states. Especially with E.Coli produced recombinant proteins, refolding analysis is often required as part of both manufacturing process analysis technology as well as quality control testing of a finished product. Interferon-α was exposed to chemical unfolding conditions at higher temperature in the presence of DTT. The mixture of intact and unfolded HuIFN-α was analyzed on reversed phase columns using the Jupiter 300 5 µm C4 and 3 µm C18 media. Well-resolved peaks on **Figure 3** represent folded and unfolded HuIFN-α protein. Comparing

chromatographic results, better resolution was achieved on Jupiter® 300 3 µm C18 versus the Jupiter 300 5 µm C4 bonded phase media (**Figure 3** Left chromatogram). Jupiter 300 3 µm C18 could detect aggregate impurities lower than 5 % as shown on **Figure 3** and **Figure 4**.

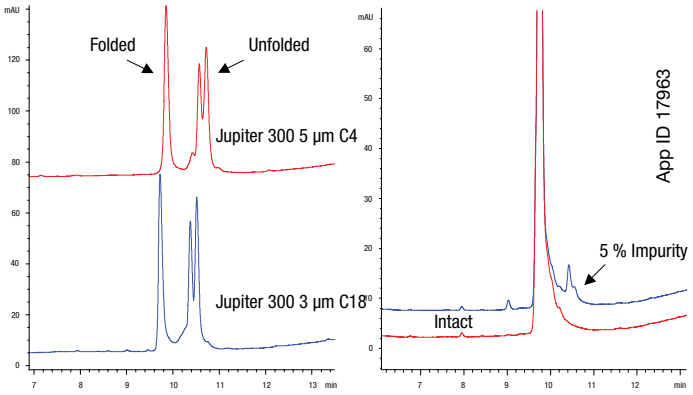

Figure 3.
Reversed phase chromatography well resolves peaks representing folded and unfolded protein HuIFN-α (mixture 50:50 v/v) on different media. Good resolution was achieved on Jupiter 300 3 µm C18 (Blue) versus Jupiter 300 5 µm (Red) C4 bonded phase media.

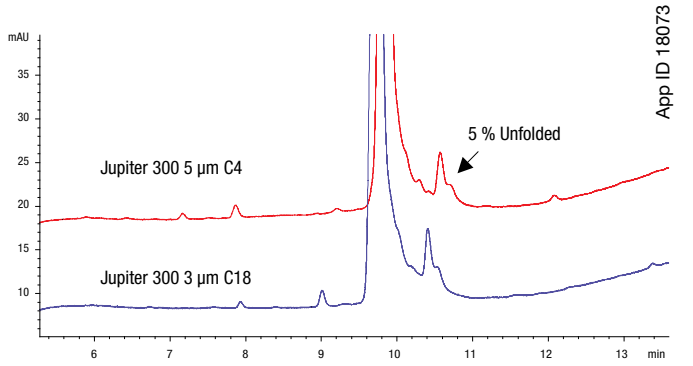

Figure 4.
Compare reversed phase separation of impurity 5 % unfolded Interferon-α protein. Good similar resolutions were achieved on selected Jupiter medias.

Monitoring a chemical degradation of biological protein as a major consequence of asparagine/glutamine deamidation and methionine oxidation was also performed with selected reversed phase media. For this intention, the intact Human Interferon-α protein was stressed with oxidizing reagents at elevated temperature for MET-oxidation and with Tris/Citrate buffer at high pH and elevated temperature for ASN- and GLN-deamidation. To demonstrate protein physical condition changes, the intact HuIFN-α and modified MET-oxidized HuIFN-α were separated on different Jupiter 300 media (**Figure 5**). For oxidized Interferon-α the Jupiter 300 3 µm C18 media outperforms the Jupiter 300 5 µm C4 media in achieving separation of the oxidized species from the intact protein. Experiments looking at interferon under deamidation conditions were performed. Interferon-α was incubated overnight under deamidating conditions and then compared to intact protein by reversed phase chromatography on the Jupiter 300 media (**Figure 6**). Results indicate that the protein is only slightly modified under such conditions, and it again appears that the Jupiter 300 3 µm C18 media provides slightly better resolution of the modified species.

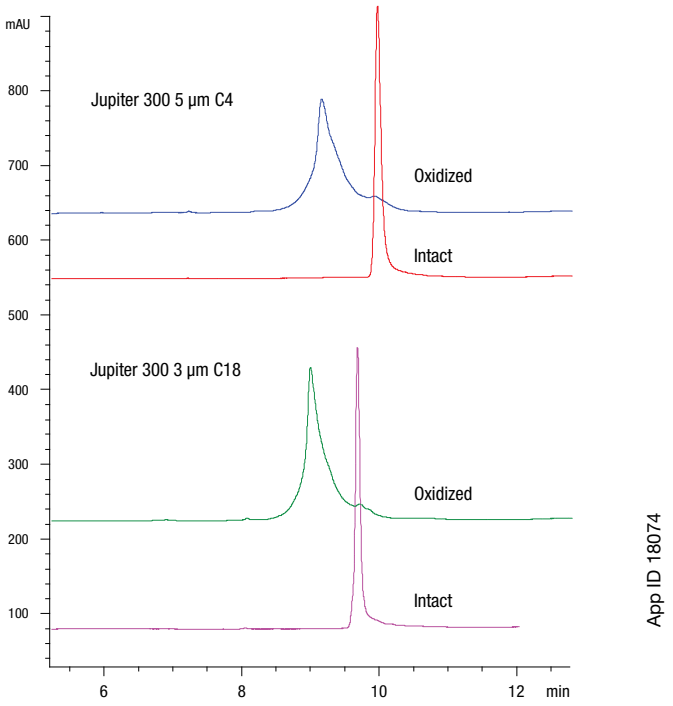

Figure 5.
Compare separations of intact HuIFN-α and modified MET-oxidized HuIFN-α (in citrate buffer pH 4.4 with 3 % H_2O^2 at 45 °C for 120 min) on different Jupiter 300 bonded phase media.

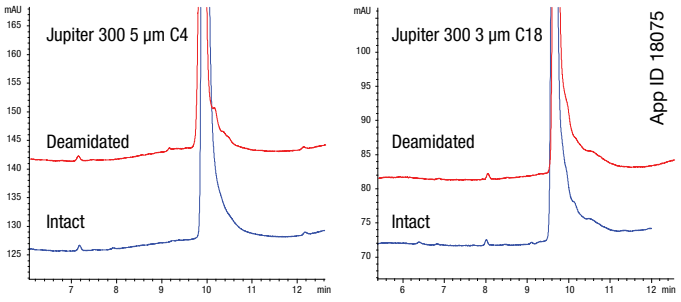

Figure 6.
Zoom view of separation of intact and modified HuIFN-α (deamidated overnight at 45 °C in Citrate/Tris buffer pH 9.0) on Jupiter 300 5 µm C4 and Jupiter 300 3 µm C18 columns. Note the tailing of the deamidated protein. Such results suggest only limited degradation under conditions tested.

Conclusions

This technical note reviews the analytical selection of reversed phase media for the stability studies of the therapeutic protein, human Interferon-α. Several modifications of the therapeutic protein including aggregation and degradation due to oxidation or deamidation are analyzed. We show that peptide mapping is a useful analytical approach to monitor protein modifications based on their peptides distribution and structural changes. Good chromatographic results were achieved on all the Jupiter® media; however it appears that for Interferon-α the Jupiter 300 3 µm C18 column outperforms the other Jupiter media for both the peptide mapping and intact protein analysis.

References:

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Ordering Information

Part No.	Description	Unit
00F-4053-B0	Jupiter 300 5 µm C18, 150 x 2.0 mm	Ea
00F-4167-B0	Jupiter 300 5 µm C4, 150 x 2.0 mm	Ea
00F-4263-B0	Jupiter 300 3 µm C18, 150 x 2.0 mm	Ea
00F-4396-B0	Jupiter Proteo 4 µm C12, 150 x 2.0 mm	Ea

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