AN AUTOMATED UPLC/MS DATA ANALYSIS USING BIOPHARMALYNX ; A CASE STUDY FOR CHARACTERIZATION OF THERAPEUTIC INTERFERON PROTEIN

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OVERVIEW

- Therapeutic Interferon proteins were analyzed to demonstrate the utility of BiopharmaLynx™ software.
- Fast data process, accurate protein MW determination and peptide mapping to confirm protein sequence were demonstrated.
- Efficient data interpretation was achieved using automated features for peptide peak annotation and data comparison.

INTRODUCTION

Interferon alpha (IFN α) proteins are a group of genetically related cytokine with a wide spectrum of biological activities including antiviral, antiproliferative, and immunomodulatory properties.[1,2] The goal of the study is to characterize and compare the recombinant Interferon α-2b proteins harvested from different cell growth conditions.

Intact Interferon protein and the enzymatically digested protein were analyzed using Liquid Chromatography-Time of Flight Mass Spectrometry (LC/TOF-MS). LC/TOF-MS has been extensively used for biotherapeutic drug development. Conventionally, LC/TOF-MS data generated for these studies is interpreted manually or being processed with a limited automation to confirm protein masses and peptide maps. They are often time-consuming processes and offer limited information due to the high complexity nature of the data.

BiopharmaLynx, a new application manager, significantly reduces data analysis time for UPLC/TOF-MS peptide mapping and intact protein analyses. It automatically annotates the peaks from LC/TOF-MS data. This software also automates batch data processing of multiple UPLC/MS experiments. The display tools for data interpretation in user friendly interface are some of the features designed to reduce the burden of data analysis.

Waters ACQUITY UPLC® system with SYNAPT™ MS System and BiopharmaLynx™ Application Manager for MassLynx™ software

METHODS

Intact Protein LC and MS conditions

Waters ACQUITY UPLC System
Waters MassPREP™ Micro Desalting column 2.1 x 5 mm
Mobile phase A : 0.1% Formic acid in water
Mobile phase B : 0.1% Formic acid in acetonitrile
Column Temperature : 80 ˚C
Gradient [3] : Total run time in 10 min
5—10 %B in 0.25 min (0.2 mL/min, waste)
10—20 %B in 0.01 min (0.2 mL/min, MS)
20—45 %B in 7.1 min (0.2 mL/min, MS)
3-sawtooth 90—5 %B in 0.5 min (0.5 mL/min, wash)

Waters LCT Premier™ ESI-TOF Mass Spectrometry
Capillary Voltage : 3.2 kV
Cone Voltage : 40 V
Desolvation Temperature : 350 ˚C
Desolvation Gas : 800 L/hr
Source Temperature : 150 ˚C
Acquisition : 600-3000 m/z

Peptide Mapping LC and MS Conditions

Waters ACQUITY UPLC System
Waters ACQUITY UPLC BEH 300 C18, 1.7 µm 300 Å
Peptide Sepration Technology, 2.1 x 150 mm column
Mobile phase A : 0.1% formic acid in water
Mobile phase B : 0.1% formic acid in acetonitrile
Flow : 200 µL/min.
Gradient : 0-50 %B over 120min.
Column Temperature : 60 ˚C
Weak wash : 95 % buffer A / 5 % buffer B
Strong wash : 20 % buffer A / 80 % buffer B

Waters SYNAPT ESI-TOF Mass Spectrometry
Capillary Voltage : 3.0 kV
Cone Voltage : 35 V
Desolvation Temperature : 250 ˚C
Desolvation Gas : 350 L/hr
Source Temperature : 120 ˚C
Acquisition : 50-1700 m/z for peptide,
Lockmass : 100 fmol/µL Glu-Fibrinopeptide B directly infused

Sample preparation

Production batches of recombinant human interferon α-2b protein were used. *A control IFN and an analyte IFN were expressed in two different cell conditions in X and Y.*
For peptide mapping, a control and one of analytes were dissolved in 50 mM ammonium bicarbonate and denatured with 0.025 % RapiGest™ SF (Waters), then reduced and alkylated with DTT and iodoacetamide (Sigma) respectively. Finally the protein was digested with trypsin (Promega).
For intact protein analysis, the samples were dissolved in 50 mM ammonium bicarbonate and directly injected into a column.

BiopharmaLynx 1.1 Application Manager

LC/MS data were processed in BiopharmaLynx 1.1. The screen captures from BiopharmaLynx browser were shown in the results section.

RESULTS

INTACT PROTEIN ANALYSIS

Figure 1. **Raw spectra** of intact IFN control and analyte

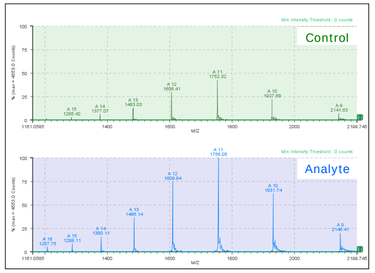


Figure 2. **Processed spectra** in mirror view;
The processed spectrum is a MaxEnt1 spectrum

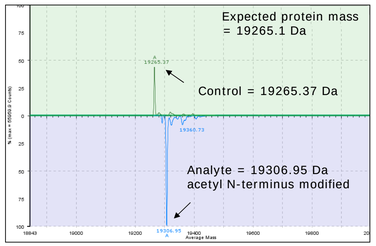


Figure 3. **Peak match data table**

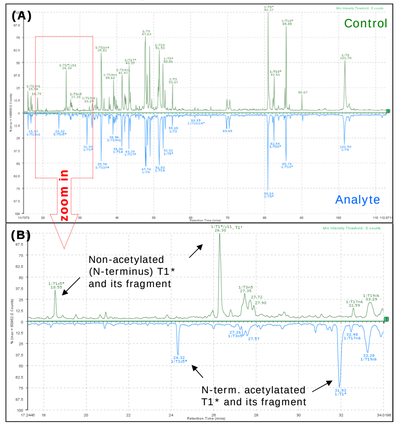
Chromatogram | Spectrum | Coverage Map | Protein Digests | Peak Match Data

	Protein	Modifiers	Calculated Protein Mass (Da)
Control	IFN-α2b		19265.1543
	IFN-α2b	Oxidation M(1)	19281.1484
Analyte	IFN-α2b	Acetyl N-TERM(1)	19307.1641
	IFN-α2b	Acetyl N-TERM(1),Oxidation M(1)	19323.1602

The intact protein analysis revealed that the control is the expected IFN and its oxidized forms. The analyte was confirmed as acetylated IFN and oxidized form of acetylated IFN.

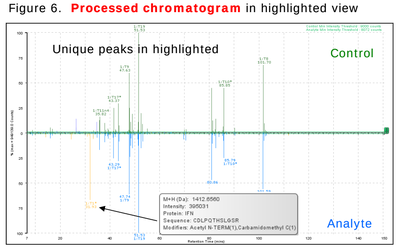
PEPTIDE MAPPING ANALYSIS

Figure 4. **Automated peak annotation** in raw TIC chromatograms in Mirror view



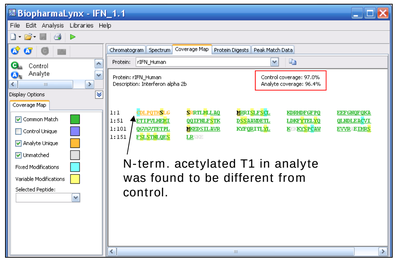
(A) Peptide maps of control and analyte in chromatogram comparison view. The tryptic peptides were annotated with protein digest fragment numbers (T_n), e.g., T1 is the first tryptic peptide from N-terminus. (B) Enlarge the area to view the differences in the control and analyte chromatograms. T1 indicated the modified form of T1. The control T1 was carbamidomethylated on N-terminus cysteine and analyte T1 was carbamidomethylated and acetylated on the same cysteine (see Figure 7 for the Interferon amino acid sequence).*

Figure 6. **Processed chromatogram** in highlighted view



The processed chromatogram provides simpler view. Each "stick" represents the summed intensity (ion counts) of all isotopic peaks for all detected charge states of that peptide over the full chromatographic elution profile.

Figure 7. **Coverage Map**



96.4 % and 97.0 % coverage were achieved in control and analyte respectively. The color coded coverage view provides additional information, such as peptides that have been matched, modified and unmatched.

Figure 5. **Peak match data table**

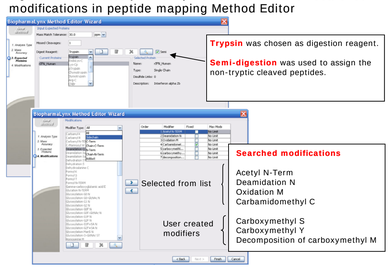
Chromatogram | Spectrum | Coverage Map | Protein Digests | Peak Match Data

Protein	Peptide	Fragment Num...	Modifiers	Calculated Peptide Mass (Da)	Control RT (Min)	Control Intensity ...	Analyte Intensity ...
IFN	CDLPQTH	1:T1c5*	Carbamidomethyl C(1)	869.3702	18.6	21847.0	
IFN	CDLPQTH	1:T1c5*	Acetyl N-TERM(1),Carbamidomethyl C(1)	911.3808			65188.9
IFN	PQTH	1:T1c5*/y4		481.2285	18.6	14858.0	
IFN	PQTH	1:T1c5*/y4		481.2285			25722.0
IFN	DLPQTH	1:T1c5*/y6					26065.9
IFN	DLPQTHSLGSR	1:T1n1					10850.0
IFN	PQTHSLGSR	1:T1n3					11578.2
IFN	SLGSR	1:T1n7			8.7	18475.0	16862.7
IFN	LGSR	1:T1n7/y4			8.7	11523.0	10756.4
IFN	GSR	1:T1n9			8.7	28282.0	29245.0
IFN	R	1:T2					
IFN	RTLMLLAQMR	1:T2-3					
IFN	TLMLLAQMR	1:T3			55.1	41334.0	17561.8
IFN	TLMLLAQMR	1:T3*	Oxidation M(1)		49.4	27360.0	24268.8

Sort
Sort By: (None)
Ascending / Descending
(None)
Protein
Peptide
Fragment Number
Start
End
Modifiers
Calculated Peptide Mass (Da)
Advanced >>

The peak match data table display contains processed results for all detected LC/MS components, including protein and peptide annotations produced by the targeted protein search results. This tabular display of peptides can be customized, sorted by any category, and have intensity thresholds applied to eliminate false identifications. In addition the table can be exported to excel spreadsheet. The control unique and analyte unique were color coded in purple and yellow in order. The peptides identified in both were displayed in green.

Figure 8. Proteolytic enzyme selection and searched modifications in peptide mapping Method Editor



Method editor can be used to batch process LC/TOF-MS peptide map data. It includes the ability to select the proteolytic enzyme and search the modifications.

CONCLUSION

- Intact protein study revealed that the analyte was acetylated IFN and its oxidized forms.
- Peptide mapping analysis reconfirmed the acetylation in the analyte sample and identified the modification site to be the N-terminus cysteine.
- The primary sequence of recombinant IFN was confirmed up to 97 % of peptide coverage. Various protein post translation modifications were identified in both control and analyte.
- BiopharmaLynx proved to be fast and efficient for automated data analysis for therapeutic protein characterization.

References

1. S. Nagata, N. Mantei, and C. Weissmann. The structure of one of the eight or more distinct chromosomal genes for human interferon-α, Nature 287:401-408 (1980).
2. D. V. Goeddel, D. W. Leung, T. J. Dull, M. Gross, R. M. Lawn, R. McCandliss, P. H. Seeburg, A. Ullrich, E. Yelverton, and P. W. Gray. The structure of eight distinct human leukocyte interferon cDNAs. Nature 290:20-26 (1981)
3. Waters Application note : 720002107EN

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