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Novel Way to Introduce the Traditional Salt Based Chromatography Technique of Ion Exchange Charged Variant Analysis of Biopharmaceutical Proteins Into High Resolution MS – CVA/MS a new multi-attribute method

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ABSTRACT

Thorough characterization of Bio-therapeutic proteins is essential at all stages of development through to manufacture and final product quality control. Each monoclonal antibody (mAb) will have several different variant forms due to multiple post translational modifications that can occur during production, purification and storage. These modifications can often alter the charge distribution on the surface of the protein which is characterized by charge variant analysis using ion exchange chromatography. Identification of structural variants is a critical challenge and mass spectrometry (MS) is used as an essential tool in the characterization and identification of these protein variants. However, the technique of ion exchange requires high salt eluents which is incompatible with MS. Structural variants exposed by these techniques must be collected separately off-line, then desalted before further characterization by MS. Here we describe a novel on-line coupling method of ion exchange to the MS instrument to allow direct characterization of mAb variants in the native form. The technique has a fast run time and greatly reduces analysis time and sample handling by avoiding fraction collection and sample desalting. The chromatographic resolution of charged variants using pH gradient elution with a novel volatile buffer preparation compares favorably with traditional salt elution. The proteins enter the Orbitrap-based MS system in the native state with a reduced charge distribution and an elevated mass to charge ratio. Variants found with this direct on-line coupling include fragments, deamidation, oxidation, glycosylation and lysine truncation in addition to the charged variant profile. The Intact mass analysis is also more accurate than figures obtained by reverse phase desalting due to the physical separation of near isobaric variants on the ion exchanger which would otherwise compromise the deconvolution.

INTRODUCTION

Charged variant analysis by ion exchange is traditionally done using salt gradient elution. However it has been shown that mAb charged variant analysis can be successfully achieved using pH gradient elution from the ion exchange column [1], The commercially available Thermo Scientific™ CX-1 pH gradient buffers [2] with the correct column produces a linear pH gradient which is easy to use. Although the salt concentrations in the eluents is greatly reduced, the buffer components used here are still not MS compatible. In this study we developed a cocktail of MS compatible buffer components to buffer in the pH range for most mAb samples, achieving the required separation of mAb charged variants directly into the MS for analysis. There are many reasons why this has been difficult to perform, including careful selection of a high resolution, low capacity column, as the column itself will act as a buffer against any pH changes. Volatile buffers do not have the high buffering capacity of the CX-1 buffer system causing delays in pH equilibration on column. Extra care was taken to develop reproducible chromatography methods for several mAb products using this pH gradient buffer system. Low gradient slopes were used in the area from pH 7 to 8, which is an area where there is no suitable volatile buffering capacity. Ion suppression of the MS signal can be caused by elevated buffer concentrations so care was also taken to balance MS sensitivity with enough buffering capacity for pH control of the column. This chromatography method will introduce the proteins into the MS in the Native form. This has the advantage of a reduced number of charged states on the protein and better spatial resolution of these charge states. Signals from the different variant forms within one charged state have more chance of being separated from the variants of the neighboring charge state.

MATERIALS AND METHODS

Equipment

Thermo Scientific™ Q Exactive™ Plus with BioPharma option, Thermo Scientific™ Vanquish™ Horizon UHPLC System, Thermo Scientific™ Acclaim™ SEC-300 column, 5µm, 4.6 x 300 mm, Thermo Scientific™ MAbPac™ SCX-10 column, 5µm, 2.1 x 50 mm; mAb samples from NIBRT;
On-line conductivity and pH monitoring was used during pH gradient optimization with the Thermo Scientific™ UltiMate™ 3000 PCM-3000 monitor.

Data Analysis

Thermo Scientific™ BioPharma Finder 2.0software, Thermo Scientific™ Chromeleon CDS 7.2, Thermo Scientific™ Xcalibur™ software v 2.2;

RESULTS

Volatile pH Gradient Optimization

On-line monitoring of the pH was used during method development to aid in trouble shooting and to determine the real pH gradient that was being produced through the column with the buffering system. Each different mAb product has a different isoelectric point and so it is essential that each gradient method produces a pH in the column that is optimized around that value. In this way robust gradient methods were developed using a single volatile buffer cocktail for several different mAb samples. This was coupled directly into the MS for charged variant analysis and identification.

Figure 1. 1a - Chromatography of Cetuximab showing the optimized gradient conditions [red dotted line] and the monitored pH profile. A low gradient slope was used to compensate for pH instability over the area between pH 7 and pH 8; 1b - Chromatography of Trastuzumab showing the optimized gradient conditions [red dotted line] and the monitored pH profile. The slopes are linear as the start conditions are above pH 8.

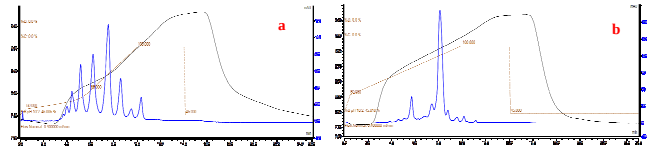
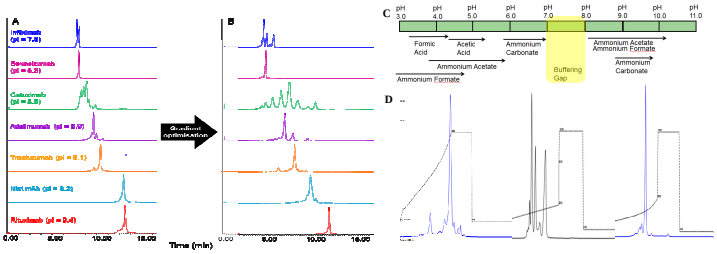
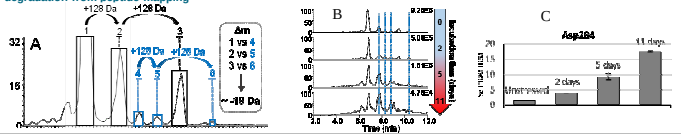


Figure 2. 2 A - Scouting chromatography runs for 7 mAb samples to derive the pH of elution; 2 B - Optimized chromatography for all 7 mAb samples, usually achieved within 1 or 2 additional runs; 2C - Available volatile buffers with the areas of buffering capacity; 2 D - Zoom of the UV trace for the optimised chromatography of 3 of the mAb samples



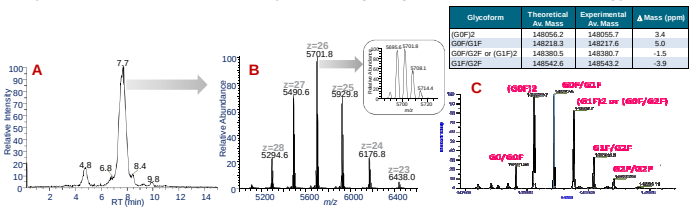
To demonstrate the global applicability of the volatile pH gradient buffer system, several mAb samples where used and gradient methods optimized for each. Figure 1a shows the technique used to control the pH over the pH 7 to 8 region with Cetuximab which elutes in this region. Trastuzumab has a higher pI and the gradient shown in 1b starts at pH 8 and avoids the need for a controlled shallow gradient. Figure 2 shows the ease of method optimization using this buffer system. A scouting run is performed from 0 to 100% eluent B over 10 minutes to determine the elution position then gradient optimization over the required pH range can be performed in the next couple of runs. Trastuzumab, Infliximab and bevacizumab results are shown in figure 2D as the UV trace with the programmed optimized gradient overlay. This shows that the chromatography is applicable to several mAb samples using this column / buffer system. The gradients for Infliximab and Bevacizumab are very similar which relates to the similar native isoelectric points of these two mAbs. Once a gradient has been optimized for one mAb, this gradient should be applicable to other mAb products with similar isoelectric points (pI).
As the protein will be in its native folded state, the charges available on the surface of the protein are much lower in number, resulting in a reduced charge distribution on the protein and an elevated mass-to-charge ratio (m/z). This results in a higher spectral resolution between m/z peaks in the native state. Modifications that could overlap with different variants from different charge states can be resolved more easily. With the volatile buffers used the resolution of the multiple variant forms for each mAb has not been compromised, showing very good resolution for all mAb samples tested. Each resolved peak will enter the MS source one at a time, providing a chromatographic separation of closely related species which could otherwise compromise the deconvolution if they were all present in the MS system at the same time.
Figure 3 shows the CVA/MS results for Adalimumab. There are clearly three lysine truncation Variants differing in mass by 128Da. However there are three additional peaks which also have a 128Da mass shift and carry an additional basic modification which causes an increase in retention time. These peaks differ in mass by 18Da from the corresponding earlier eluting lysine truncation peaks. They were found to be succinimide Asp containing versions of the lysine truncation peaks. This is a known degradation route for this protein and can be confirmed by the increase in these variant forms on forced degradation studies shown in figure 3B. All this information can be gained from one LCMS run without any sample preparation. To determine these attributes traditionally would require peptide mapping in addition to charged variant analysis. Peptide mapping has been used here to confirm the presence of the variants in the sample. The direct coupling to the Q Exactive Plus hybrid quadrupole Orbitrap mass spectrometer allows for the acquisition of accurate mass data to identify the charge variants present in the sample.

Figure 3. Optimized chromatography for Adalimumab showing the identification of several of the variants: A - BPC for Adalimumab by CVA/MS, B - Time course of forced degradation with CVA/MS,, C - Increase in Asp 284 during the forced degradation from peptide mapping



As the separated variants enter the MS system individually in a chromatographically purified form from the ion exchange column the mass accuracy is improved. This can be seen in figures 4 and 5 where the experimental mass is compared to the theoretical.

Figure 4. A - Base peak chromatogram (BPC) for Trastuzumab; B - Full MS spectrum with charge envelope for trastuzumab main peak at RT 7.7 min; C - Deconvoluted mass spectrum showing average masses for the four most abundant glycoforms

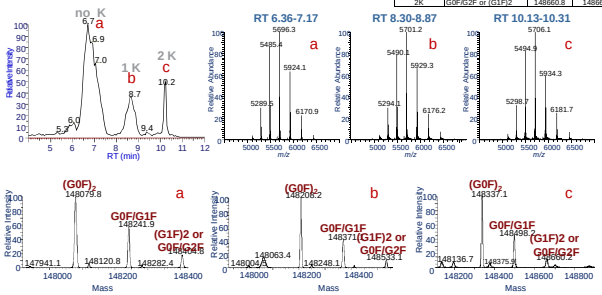


Glycoform	Theoretical Av. Mass	Experimental Av. Mass	Δ Mass (ppm)
(G0F)2	148056.2	148055.7	3.4
G0F/G1F	148218.3	148217.6	5.0
G0F/G2F or (G1F)2	148380.5	148380.7	-1.5
G1F/G2F	148542.6	148543.2	-3.9

Trastuzumab in figure 4 shows two main peaks in the BPC with a clear, well resolved mass charge envelope. The deconvoluted average mass values correspond well to the theoretical values. The peak eluting at 4.8 minutes represents a deamidated form which averages experimentally at 0.8 Da bigger than the main peak corresponding to the theoretical mass difference of 0.98 for a deamidation event. The glycoforms present under a single charge state can be seen clearly from the raw data in the zoom in figure 4b. The deconvoluted values in Figure 4c exhibit close correlation to the theoretical mass values expected.

Adalimumab (Humira™, Abbvie Inc.) data is shown in figure 5 exhibits three major charged variant forms which can be identified as lysine truncation variants. The deconvoluted mass for all three variants show the mass difference corresponding to additional lysine residues. As lysine carries a positive charge the addition of each lysine to the end of the heavy chain increases the retention on the cation exchange column.

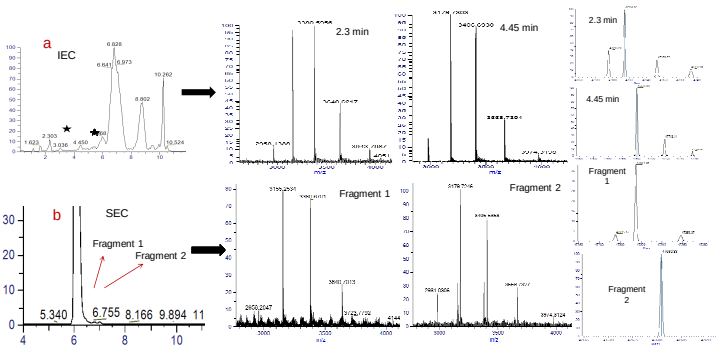
Figure 5. BPC for Adalimumab showing three main peaks a, b, c. followed by the Full MS spectrum with charge envelope for each peak and the deconvoluted mass spectra for each peak showing the average masses for the three most abundant glycoforms for each lysine truncation variant.



Lys variant	Glycoform	Theoretical Av. Mass	Experimental Av. Mass	Δ Mass (ppm)
no K	(G0F)2	148080.2	148079.8	2.6
no K	G0F/G1F	148242.3	148241.9	2.9
no K	G0F/G2F or (G1F)2	148404.5	148404.8	-2.3
1K	(G0F)2	148208.4	148208.2	1.0
1K	G0F/G1F	148370.5	148371.1	-4.1
1K	G0F/G2F or (G1F)2	148532.6	148533.1	-3.1
2K	(G0F)2	148336.5	148337.1	-3.9
2K	G0F/G1F	148498.7	148498.2	3.2
2K	G0F/G2F or (G1F)2	148660.8	148660.2	4.1

The experimental deconvoluted mass values again correspond favorably with the theoretical values giving unambiguous peak assignments. Two of the earlier peak assignments for the Adalimumab ion exchange chromatography show the possible presence of fragments. This is confirmed by size exclusion chromatography using the Acclaim SEC 300 column with the same buffers.

Figure 6. 6a - BPC for IEC of Adalimumab followed by the Mass spectra for the peaks at 2.3 and 4.4 minutes; 6b - BPC for the SEC of Adalimumab followed by the mass spectra for the fragment peaks eluting after the full sized mAb



Size exclusion chromatography is another method which allows the introduction of protein samples into an MS source in the native folded state. The separation can be used to both desalt the sample and separate aggregates and fragments. The separation buffer must be optimized for MS sensitivity by reducing the salt content with a column that has reduced secondary interactions, maintaining chromatographic integrity. SEC is not a concentration technique, as such sample loading is more limited than with ion exchange, as can be seen from the Improved sensitivity with CVA/MS. The results confirm that the same fragments are seen in both SEC and IEC. The higher loading capabilities of the pH gradient ion exchange chromatography technique allows significantly more sensitivity to characterise low level variant species.

CONCLUSIONS

- Ion Exchange has been successfully interfaced directly to MS for charged variant analysis and direct identification.
- The method requires a volatile buffer cocktail with a carefully selected low capacity, high resolution ion exchange column.
- The system has global applicability demonstrated with several mAb samples using gradients optimized to the pI of the protein.
- Multiple attributes can be determined with a single direct injection of sample including; Intact mass, CVA pattern, glycan distribution, lysine truncation, deamidation, succinimide Asp modification and fragment analysis.
- From a direct injection of product without any sample preparation, multiple attributes have been determined which would otherwise have used several chromatography methods with different sample preparation protocols. This is a huge saving on resources and time.

REFERENCES

1. Farnan D, Moreno G.T. Analytical Chemistry 2009, 81(21), 8846–57
2. Thermo Scientific CX-1 pH Buffer product manual, P/N 065534-01 June 2013

TRADEMARKS/LICENSING

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