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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. 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. Variants must be collected separately off-line, then desalted before further characterization by MS. Here we describe on-line coupling of ion exchange and SEC to allow direct MS characterization of mAb variants in the native form. The technique has a fast run time and avoids fraction collection with sample desalting. The chromatographic resolution using the pH gradient volatile buffer preparation is extremely high. Protein variants 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 method 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. The sensitivity is also increased compared to SEC.

INTRODUCTION

It has been shown that mAb charged variant analysis can be successfully achieved using pH gradient elution from the ion exchange column [1], Commercially available Thermo Scientific™ CX-1 pH gradient buffers [2] produce a linear pH gradient which is easy to use, however the buffer components used 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 that allows sufficient pH control for chromatographic stability and optimum sensitivity for MS analysis. This balance is not easy to achieve and includes careful selection of a high resolution, low capacity column, essential for the method stability. The charged moieties on the column itself will act with a significant buffering capacity against any pH changes. Volatile buffers do not have the high buffering capacity of the CX-1 buffer system creating delays in pH equilibration on column. Extra care was taken with on-line pH monitoring to develop reproducible chromatography methods with this pH gradient buffer system. Low gradient slopes were used in the area from pH 7 to 8 where there is no suitable volatile buffering capacity. This chromatography method used here 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.

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 using the Thermo Scientific™ UltiMate™ 3000 PCM-3000 monitor.

Data Analysis

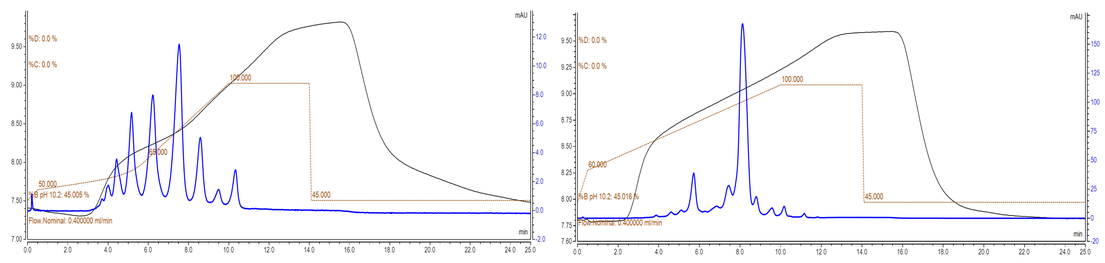
Thermo Scientific™ BioPharma Finder 3.0 software, Thermo Scientific™ Chromeleon CDS 7.2, Thermo Scientific™ Xcalibur™ v 2.2.

RESULTS

Volatile pH Gradient Optimization

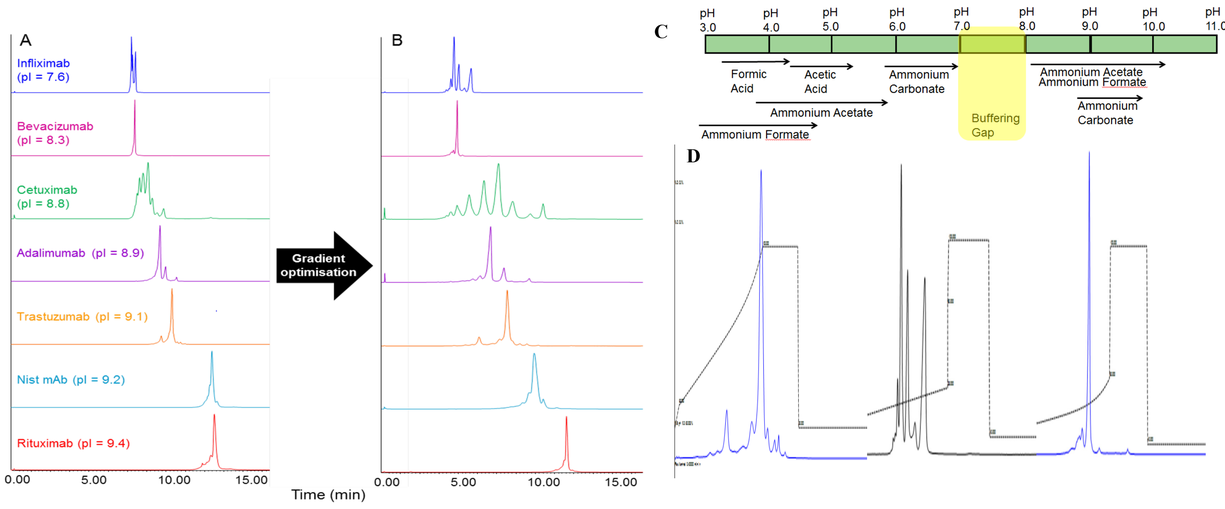
On-line monitoring of the pH was used during method development to aid in trouble shooting and to determine the real pH produced through the column in response to the programed gradient. Each mAb product has a different isoelectric point and so it is essential that each gradient method produces a pH on 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.



a b

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 optimized chromatography of trastuzumab, infliximab and bevacizumab samples

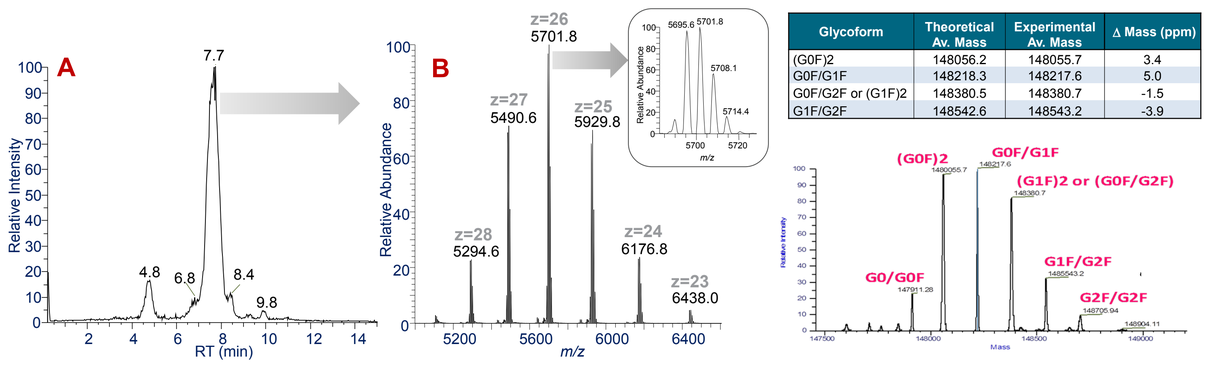


To demonstrate the global applicability of the volatile pH gradient buffer system, several mAb samples where used and gradient methods optimized for each. Figure 1a shows the control of the pH over the pH 7 to 8 region with Cetuximab. Trastuzumab [1b] has a higher pI with the gradient starting at pH 8, the pH profile here shows a good correlation to the programed gradient.

Figure 2A,B shows the ease of method optimization, 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 within the next couple of runs. The high resolution separations achieved for trastuzumab, Infliximab and bevacizumab are shown in figure 2D as the UV trace including the programmed optimized gradient. This demonstrates the chromatography is applicable to several mAb samples using this column / buffer combination. Once a gradient has been optimized for one mAb, this gradient should be applicable to other mAb products with similar native isoelectric points (pI). It must be pointed out that the native pI may be different from a value calculated from the sequence .

Trastuzumab in figure 3 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 glycoforms present in each charge state can be seen clearly from the raw data in the zoom in figure 3b. The deconvoluted values [Figure 3c] exhibit close correlation to the theoretical mass values expected. The peak eluting at 4.8 minutes is a deamidated variant with an experimental mass 0.8 Da bigger than the main peak corresponding to the theoretical mass difference of 0.98 for a deamidation event. It must be noted that this would not be seen by infusion or desalting.

Figure 3. 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



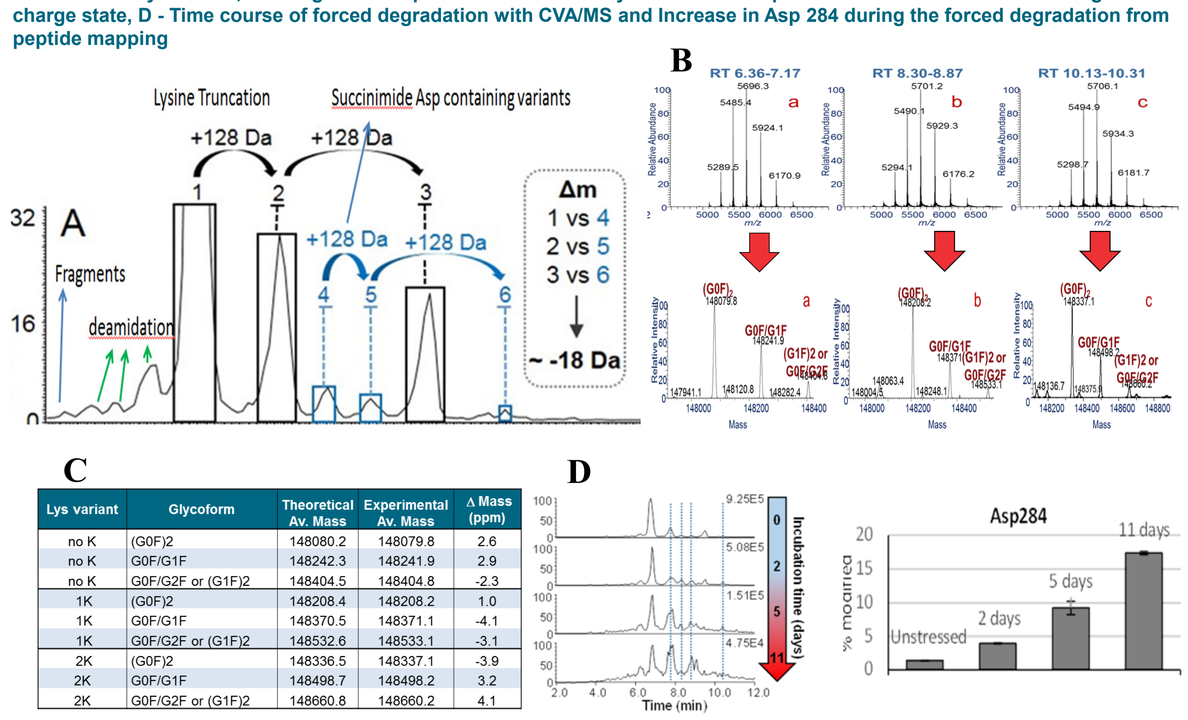
Glycoform	Theoretical Av. Mass	Experimental Av. Mass	Δ Mass (ppm)
(G0F)2	148056.2	148055.7	3.4
G0F/G1F	148218.3	148217.6	5.0
G0F/G2F or (G1F)2	148380.5	148380.7	-1.5
G1F/G2F	148542.6	148543.2	-3.9

Figure 4A shows the CVA-MS results for Adalimumab (Humira™, Abbvie Inc.) the data shows three major charged variant forms which can be identified as lysine truncation variants differing in mass by 128 Da. 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 giving a further indication of identity. The high quality of the individual charged envelopes and deconvolution are shown in figure 4B. As the separated variants enter the MS system individually the mass accuracy is improved. This can be seen in figure 4C where the experimental mass is compared to the theoretical.

There are three additional peaks which have a 128 Da mass shift [4A blue] and carry an additional basic modification which causes a further increase in retention time. These peaks all differ in mass by 18 Da from the corresponding earlier eluting main lysine truncation peaks. They were found to be succinimide Asp containing versions of the lysine truncation peaks. This known degradation route can be confirmed by the increase in these variant forms on forced degradation studies shown in figure 4D. The site of modification was identified as Asp 284 by peptide mapping, which shows the same increase in relative abundance over the time course of forced degradation [Figure 4D].

We also see fragments and deamidation variants as early eluting peaks. All this information is obtained from one 15 minute LCMS run without any sample preparation. Fraction collection and peptide mapping has been used here to confirm the identity of the variants in the sample. The direct coupling of the native charged variant separation to the Q Exactive Orbitrap mass spectrometer with BioPharma option then provides accurate native mass data to identify the charge variants present in other samples without the need for peptide mapping.

Figure 4. Optimized chromatography for Adalimumab showing the identification of several of the variants: A - BPC for Adalimumab by CVA/MS, B Charged envelope for the three main lysine truncation peaks with deconvolution of the highest charge state, D - Time course of forced degradation with CVA/MS and Increase in Asp 284 during the forced degradation from peptide mapping

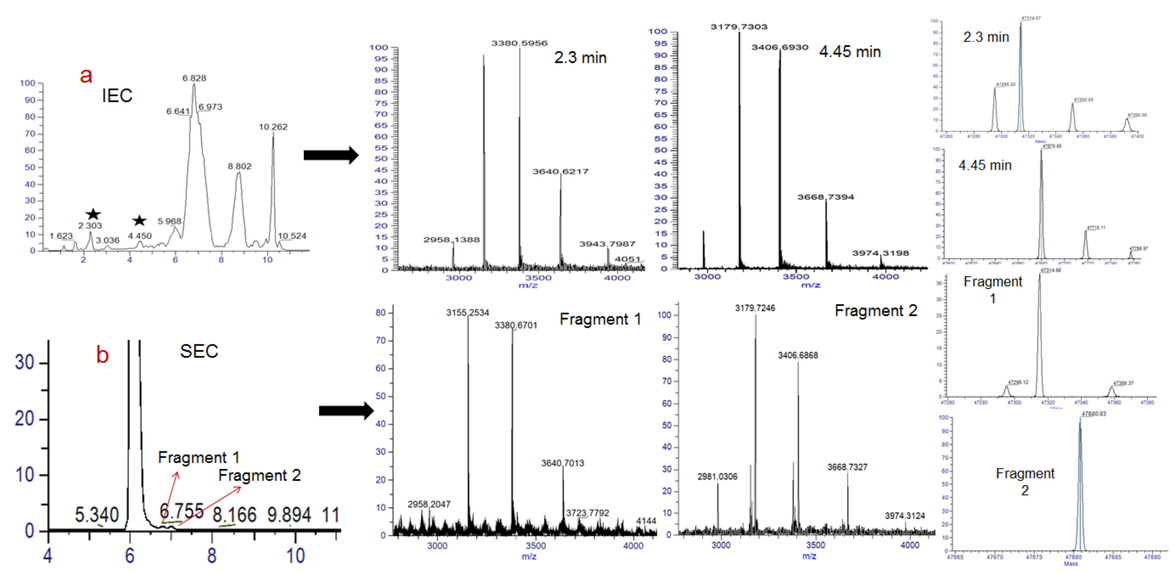


Lys variant	Glycoform	Theoretical Av. Mass	Experimental Av. Mass	Δ Mass (ppm)
no K	(G0F)2	148080.2	148079.8	2.6
no K	G0F/G1F	148242.3	148241.9	2.9
no K	G0F/G2F or (G1F)2	148404.5	148404.8	-2.3
1K	(G0F)2	148208.4	148208.2	1.0
1K	G0F/G1F	148370.5	148371.1	-4.1
1K	G0F/G2F or (G1F)2	148532.6	148533.1	-3.1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

As the charges available on the surface of the native protein are lower in number, there is 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. Each resolved peak will enter the MS source one at a time, providing separation of the closely related species which could otherwise compromise the deconvolution if present in the MS system at the same time. This also helps facilitate the higher resolution settings used in this analysis. For this reason we are seeing variants such as deamidation which only has a mass shift of 1Da. The shift in retention time of a deamidation variant due to the loss of a positive charge also helps confirm the identification. These identifications cannot be achieved with infusion experiments, reverse phase denatured intact or SEC native intact analysis.

Two of the earlier peak assignments for the Adalimumab ion exchange chromatography show the possible presence of fragments. This can be confirmed by size exclusion chromatography using the Acclaim SEC 300 column with the same buffers. 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 and using a column that has reduced secondary interactions to maintain 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 in the comparison of the results in figure 5. 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 these and other low level variant species. The resolution of the fragments is also much improved with CVA-MS.

Figure 5. 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



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.

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TRADEMARKS/LICENSING

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