Comprehensive Characterisation of Monoclonal Antibodies Using Charge Variant Analysis Coupled to High-Resolution Mass Spectrometry

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Native mass spectrometry (MS) is an increasingly important technique for the characterisation of recombinant protein-based biopharmaceutical products such as monoclonal antibodies (mAbs), offering several benefits over MS workflows that involve protein denaturation steps. However, coupling native MS with established protein separation methods like cation exchange chromatography for charge variant analysis (CVA) has traditionally proven to be challenging. This article highlights how modern high-resolution mass analysers with extended mass capabilities are supporting the confident characterisation of biopharmaceutical products and details a global CVA-MS method for the multiparameter analysis of complex protein therapeutics.

Introduction

Thanks to their impressive specificity and safety profile, mAb-based drugs have increased steadily in use over the past 30 years to become one of the major classes of biotherapeutics [1]. An extensive range of mAb products has been approved for the treatment of a variety of conditions, including cancer as well as autoimmune and inflammatory diseases [2].

mAbs, like other recombinant protein therapeutics, are large, complex molecules that are produced using biological-based manufacturing techniques; given their structural complexity, extensive characterisation during production, purification and storage is an important regulatory requirement. Various post-translational modifications, including deglycosylations and deamidations, can impact on the activity and stability of biotherapeutic proteins, potentially putting the safety and efficacy of these products at risk [3-5]. Due to the inherent complexity caused in part by the biological production within cells, there are many attributes that need to be monitored and therefore a wide range of analytical techniques employed [6].

Many post-translational modifications result in changes to the charge distribution on the surface [7] of the protein, with cation exchange chromatography coupled to UV detection being widely used for the CVA of mAbs, as it enables the precise separation of variants that differ by just one charged residue. The recent trend towards the use of pH gradients to control protein elution has further enhanced the capabilities of this valuable technique [8-10].

Meanwhile, native MS has emerged as a powerful tool for the characterisation of recombinant protein-based therapeutics, offering several advantages over MS workflows that require denaturation of the protein prior to analysis. As native MS enables proteins to be studied under conditions that avoid the use of acidic or organic solvents, the protein analytes accept fewer charges during the ionisation process, helping to maintain the three-dimensional structure of the protein, improve spectral resolution and increase the breadth and depth of structural information that can be obtained.

To unlock the full potential of native MS for the CVA of mAbs, this technique should be paired with powerful separation technologies. However, coupling native MS with cation exchange chromatography has traditionally proven to be challenging, as the high salt concentrations in the mobile phase used for protein separation significantly complicates, and thereby precludes, detection by MS due to the high concentrations of salt used in the eluent system.

The advent of high-resolution mass analysers with extended mass capabilities and use of pH gradient eluent systems based on volatile buffers is helping to overcome this challenge, and CVA methods employing MS detection have recently been reported [11-13]. Herein, the results of a combined CVA-MS method for the multiparameter analysis of seven mAbs are presented. mAbs with a broad range of isoelectric point (pI) values were chosen to demonstrate the global applicability of the method.

Experimental

Materials

Bevacizumab, cetuximab, infliximab, rituximab and trastuzumab were provided by the Hospital Pharmacy Unit of the University Hospital of San Cecilio in Granada, Spain. Adalimumab was provided by St. Vincent's University Hospital in Dublin, Ireland, and the mAb reference material RM8671 was purchased from the National Institute of Standards and Technology (NIST) in Gaithersburg, MD, USA. Between 50 and 100 µg of each mAb in a buffer solution were injected in each run, without any additional sample preparation steps. For biological samples where matrix effects must be accounted for some additional sample preparation steps were performed as required.

LC-MS conditions

CVA analysis was performed using a Thermo Scientific Vanquish Flex Binary UHPLC system coupled to a Thermo Scientific Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer equipped with the BioPharma option to extend the mass range to m/z 8000. MS conditions are shown in *Table 1*.

Table 1. Tuning and method parameters for MS analysis.

Spray voltage	3.6 kV
Capillary temperature	275°C
Sheath gas	20 arbitary units
Aux gas	5 arbitarty units
Probe heater temperature	275°C
S-Lens PR level	200
HMR mode	On
Trapping gas pressure setting	1
Polarity	Positive
In-source CID	150 eV
Microscans	10
Resolution (@ m/z 200)	17,500; 35,000; 70,000
AGC target	3e6
Maximum IT	200 ms
Scan range	2500-8000 m/z
Spectrum data type	Profile

A Thermo Scientific MAbPac SCX-10 RS 5 µm 2.1 × 50 mm column was used for the separation, with a flow rate of 0.4 mL/min, column temperature of 30°C and injection amount of 50-100 µg. Mobile phase A consisted of 25 mM ammonium bicarbonate and 30 mM acetic acid in water (final unadjusted pH 5.3). Mobile phase B consisted of 10 mM ammonium hydroxide in water (final unadjusted pH 10.9). Mobile phase gradients for CVA analysis are shown in *Table 2*. Each sequence incorporated a re-equilibration step involving a flush using 100% mobile phase A for 2 min followed by equilibration to the starting conditions for 8 min.

Table 2. Mobile phase gradients for CVA of seven mAbs.

mAb	Time [min]	%B	Curve
Infliximab	0	30	
	10	55	5
Bevacizumab	0	35	
	10	60	5
Cetuximab	0	30	
	0.5	40	5
	3.5	42	7
	7	55	5
	10	100	5
Adalimumab	0	40	
	10	100	5
Trastuzumab	0	40	
	10	100	5
Rituximab	0	85	
	10	100	5
NIST mAb	0	90	
	10	100	5

Data analysis

Deconvoluted spectra for each sample were obtained using Thermo Scientific BioPharma Finder 3.0 software for native intact mass analysis.

Method Development and Optimisation

The separation of the seven mAbs was efficiently achieved using a CVA method employing a pH gradient. Notably, the method did not involve any sample preparation steps, for example filtering or purification, which resulted in a shorter workflow that reduced the potential for method variability; both of which are important considerations within the routine biopharmaceutical testing environment.

The CVA approach adopted in this study involved the application of a pH gradient to the chromatography system over the course of each analytical run. When the pH of the mobile phase exceeded the pI of the charge variant, the protein was no longer retained and was therefore eluted from the column. Volatile buffers were used to minimise the presence of salts in the eluent system, both ensuring compatibility with MS, and maintaining the proteins in their native form, preserving their three-dimensional structure and resulting in a smaller exposed surface area to accept charges. Given the reduced number of charged states (with lower charge values) detected at higher m/z values, the extended mass range offered by the MS system was essential.

One of the key challenges traditionally encountered when working with volatile MS-compatible buffers is the lack of reagents to provide suitable on-column buffering capacity between pH 7 and 8. This issue often results in an uncontrolled jump in pH for gradient methods that span this range, causing proteins and their variants with a pI value in this region to elute at the same retention time. To avoid this issue, separation methods encompassing this pH range must employ a shallow curved gradient at this point to deliver a near-linear on-column pH gradient. To support the development of suitable pH gradients, an on-line pH and conductivity monitor was used to measure the actual on-column pH resulting from the gradients employed.

The non-porous resin-based analytical column used in this study worked well in combination with the chromatographic conditions, offering high-resolution mAb separations. The hydrophilic layer surrounding the column's polymer beads eliminated hydrophobic interactions with the resin to enable highly efficient separations [12]. Additionally, the column dimensions were well matched with the relatively weak buffering capacity of the volatile buffers, facilitating the use of a low flow rate for HRMS data acquisition and enabling rapid pH gradient generation and system re-equilibration.

The results of the shallow curved pH gradient on the separation of cetuximab over the pH range 7 to 8 are shown in *Figure 1*. The shallow gradient successfully prevents a sudden increase in pH, resulting in a relatively linear on-column pH profile and effectively separating the cetuximab charge variants, as evidenced by the eight peaks observed in the chromatogram over the optimised gradient. In the absence of the shallow curved gradient, the pH was uncontrolled and increased rapidly over the pH range 7 to 8, resulting in co-elution of the charge variants.

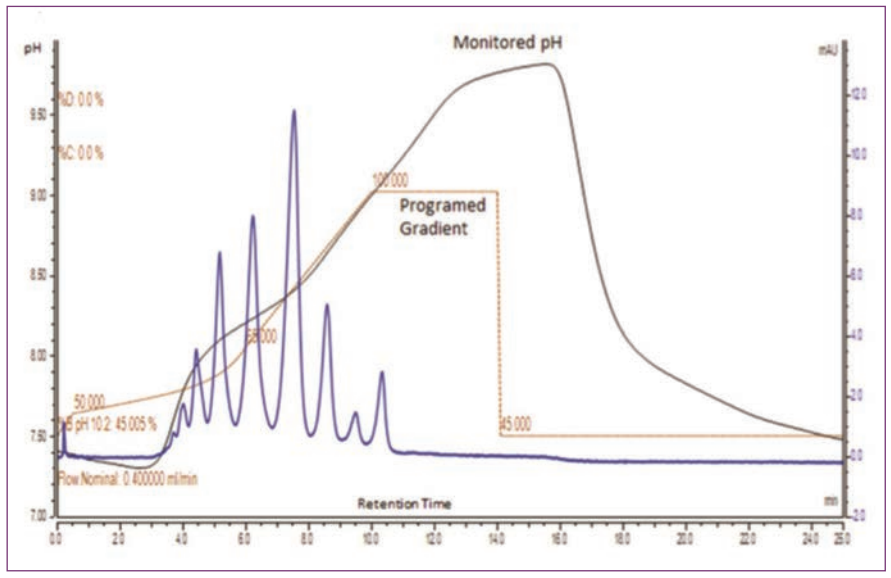

Figure 1. Programmed gradient and actual on-column pH gradient for the analysis of cetuximab.

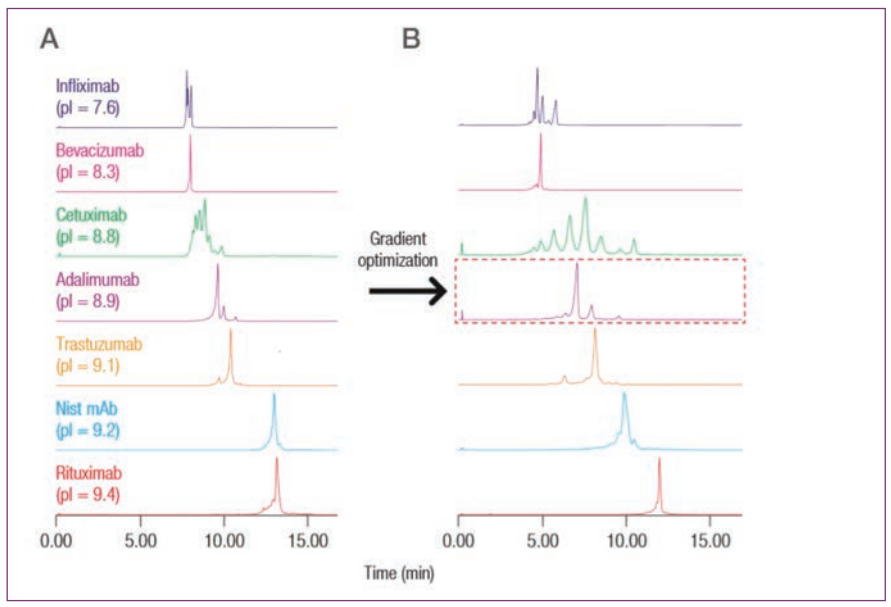

Figure 2. Results of gradient optimisation for CVA-MS analysis of seven mAbs; (A) initial gradient from 0 to 100% mobile phase B, (B) optimised gradients.

Figure 2 highlights the results of gradient optimisation for the analysis of the seven mAbs, each with varying pIs. In each case, a linear gradient from 0 to 100% mobile phase B was initially employed. Gradient methods for each mAb were then quickly optimised over two or three injections, highlighting the speed of method development using the separation system.

To further improve method performance, MS settings were optimised to eliminate isobaric interference. The high-resolution Orbitrap system's extended mass range was utilised to detect the mAb lower charge states at the higher m/z values afforded under native conditions. The increased spatial resolution between charged states was important for these highly complex molecules, as two adjacent charge states may overlap in the charge envelope of a denatured spectrum, making annotation more challenging and less confident. Although this issue can be resolved when analysed under native conditions, reversed-phase or size exclusion chromatography techniques do not separate charge variants prior to MS analysis. As a result, the variants will enter the mass spectrometer simultaneously, resulting in a more complex analysis and increasing the risk of missing low abundance variants.

Employing charge variant separation prior to MS analysis enabled a more accurate and sensitive analysis. *Figure 3* highlights data for adalimumab acquired at a resolution of 17,500, 35,000 and 70,000 (determined at m/z 200). In each case, the charge envelope of the main peak in the chromatogram is shown in more detail *(Figure 3B)*. Although increases in resolution resulted in a decrease in total signal intensity, variants with similar m/z values (with a greater risk of co-eluting) were more likely to be distinguished *(Figure 3C)*. These results highlight how the enhanced resolution offered by the system support the confident identification of variants, while the ability to apply lower resolution settings when necessary can benefit lower abundance species.

Global mAb Characterisation

One of the critical quality attributes often assessed in mAb characterisation workflows is the relative abundance of N-glycoforms. N-glycans present on the structure of mAbs play a key role in maintaining the protein's structural stability. Consequently, deglycosylation can make products more vulnerable to aggregation and impact on the intended mechanism of action [3,4].

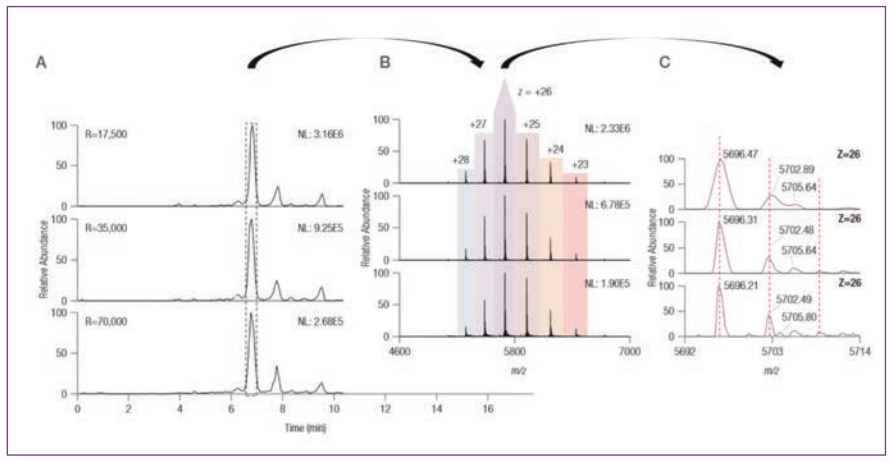

Figure 3. (A) Base peak chromatogram of 100 µg of adalimumab at resolutions of 17,500, 35,000, and 70,000. (B) Charge envelope of the main variant peak (MS signal intensities for the base peak chromatogram and the charge state envelopes are shown). (C) Magnification of the +26 charge state showing the distribution of glycoforms and a co-eluting species.

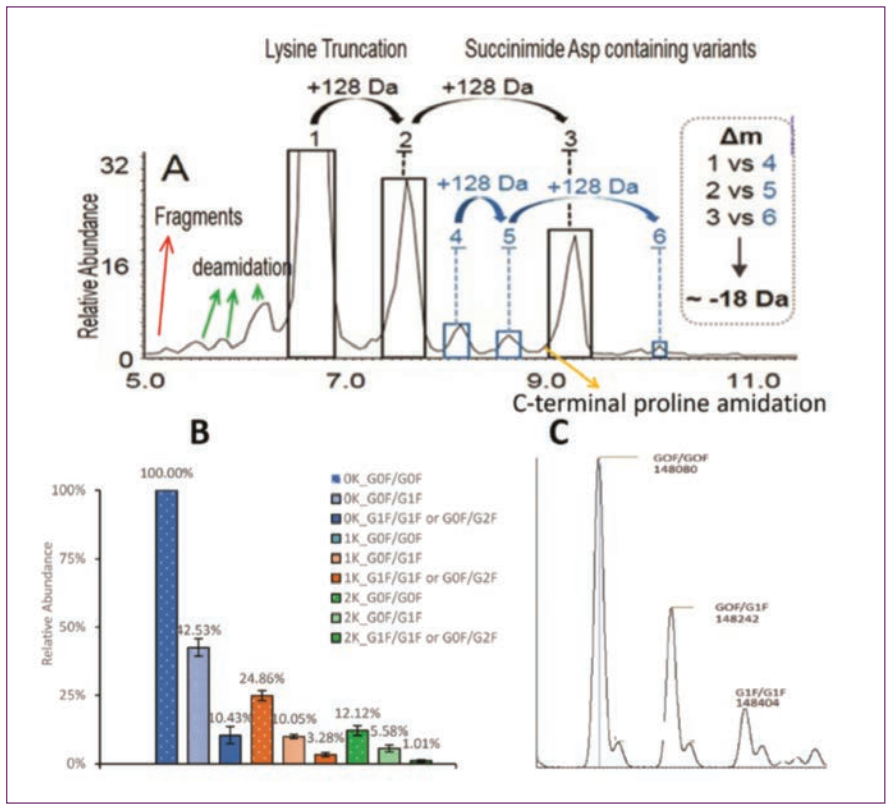

Figure 4. (A) Base peak chromatogram of 100 µg of adalimumab acquired at a resolution of 35,000. (B) Glycan ratio for each main lysine variant. (C) Deconvoluted annotated spectrum of the main peak.

The CVA-MS method used in this study enabled the quantitative and qualitative analysis of all major glycoforms of the main charge variants. The only glycosylations resulting in shifts in retention time by cation exchange chromatography were forms containing sialic acids, as observed for the cetuximab sample, which caused the protein pI to decrease and elute earlier from the column. As the relative abundance of specific glycans can differ significantly between mAbs, the annotation of mAbs by intact mass analysis can be compromised by the co-elution of other similar proteoforms. The excellent purity of the peaks obtained by the CVA-MS separation method enabled high accuracy mass measurements of each peak, supporting the confident annotation and relative quantitation of glycoforms for each charge variant. This method offers significant benefits over traditional CVA methods paired with conventional detection techniques, as co-eluting variants can be distinguished using the difference in intact mass measurement.

Figure 4 highlights how the method was used to determine several other key characteristics, using adalimumab as a representative example. The separation and identification of various lysine truncation variants, each differently glycosylated, is shown in *Figure 4A*. Other species, including fragments, deamidated variants and succinimide aspartic acid-containing variants, could also be identified within the same analytical run. Peaks 1–3 represent the main lysine variants, while peaks 4-6 correspond to the succinimide aspartic acid-containing versions of these lysine truncated forms. Lysine truncation variants that also contained a succinimide modification of an aspartic acid residue were associated with shorter retention times and a difference in mass of 18 Da compared with the unmodified lysine variant. The peaks labelled with green arrows correspond to deamidated forms, and the early eluting peaks labelled with a red arrow were confirmed as fragments resulting from cleavage at the hinged region of the mAb. The glycan ratio for each lysine variant is shown in *Figure 4B*, highlighting the identical distribution of glycans at high mass accuracy, while the deconvoluted annotated spectrum of the main peak is shown in *Figure 4C*.

Conclusion

Using the globally applicable CVA-MS method presented here, the comprehensive characterisation of seven mAbs spanning a range of pI values was achieved. The method required no sample preparation and enabled the measurement of multiple attributes from single direct injection, significantly improving the speed and efficiency of comprehensive characterisation. The optimised volatile buffer mixture [13], high-resolution ion exchange column, and extended mass range of the mass spectrometer were key to achieving these results. It is hoped that CVA-MS methods based on this approach will accelerate the confident multi-parameter analysis of mAb charged variants in routine biopharmaceutical characterisation workflows.

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1. National Institute for Bioprocessing Research and Training (NIBRT), Dublin, Ireland.
2. Thermo Fisher Scientific, Hemel Hempstead, UK.
3. Thermo Fisher Scientific, Germering, Germamy.

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