New Innovations in UHPLC and multidimensional Workflows for the Characterisation of Bio-Therapeutics

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New Challenges in Characterisation of Protein Therapeutics

The bio-therapeutic protein market is growing at a tremendous speed. There are many more Bio-Therapeutics in the clinical pipeline some of which have additional characterisation requirements. These large bio-molecules are very complicated and require multiple characterisation methodologies. These factors have created a requirement for speed, high resolution and robustness of analysis.

Monoclonal antibodies remain the most popular protein bio-therapeutic by a good margin due to the effectiveness and specificity of this type of molecule, particularly in the treatment of cancer where there is a need to make the difficult distinction between normal host and tumour cells. The effective targeting of MAb's towards infected cells has given rise to the use of antibody drug conjugates where a toxic small drug molecule can be linked to the antibody to direct the toxic payload to the required area of treatment. Drugs of this toxicity could not be used systemically due to the massive side effects caused by off target action. Directed to the correct place of action by the MAb produces a very effective way of removing diseased cells. This success adds to the numbers of large bio-molecules that require extensive characterisation. This bottleneck for analysis has led to many advances in hardware and chemistries to provide more global methods to cope with the high throughput without sacrificing the high resolution required to produce safe drugs with high efficacy.

Changes in Analysis

MAb characterisation requires a variety of chromatography techniques. To improve these analytical requirements would include new chemistries to test for the presence of aggregates, fragments, glycosylation variants and N-terminal truncation [1, 2, 3]. In addition to increasing the speed of analysis to keep up with the high throughput there is also a move to global methodologies that can be used as a template for any new drug candidate. For example, in the past we have seen ion exchange variant analysis with 60 minute run times that need to be optimised for each different MAb [4, 5]. The optimisation of these methods requires screening for the best pH, best column as well as tight optimisation of the final gradient. With so much optimisation on a long method time this could take weeks to finalise. The move to more global pH gradients which can be used as a template for any MAb reduces method optimisation times dramatically [4]. The added advantage of being able to use shorter columns with smaller particle sizes also reduces run times from 60 minutes to as low as 2 minutes without any sacrifice on resolution. Global methods for bio-therapeutic proteins now includes size exclusion chromatography [SEC] for aggregates, Ion exchange for charged variants, Hydrophobic Interaction Chromatography [HIC] for conformational change and drug antibody ratios [DAR] on ADC's, titre analysis with Protein A, peptide mapping and intact protein and fragment analysis with reverse phase. All these methods can be utilised on any MAb and each has the capability to show up a different type of variant which may require fractionation for further analysis. With all of these template methods available a new breed of biocompatible UHPLC systems capable of multidimensional analysis and fractionation are needed. UHPLC has become commonplace with small molecule drug analysis so it is only a natural progression that the same pharmaceutical industry which pioneered this, would have the same drivers to transfer these techniques to large bio-therapeutic molecule characterisation [6]. The requirements to do this involve a new breed of column chemistries coupled to UHPLC systems that can handle the harsh corrosive eluents that come with ion exchange, affinity capture, SEC and HIC chromatography. Fractionation of variant peaks from a 5 minute UHPLC column run also requires advances to high resolution collection of unknown peaks that are only a few seconds wide. The pressure to move to UHPLC of large molecules has encouraged instrument manufacturers to produce such systems with chemistries to go with them.

Trypsin Digestion

There is no point in developing fast analytical techniques if the sample preparation required prior to analysis is difficult and time consuming. Trypsin digestion of proteins required before peptide mapping is a good example of this. The process requires several of the reagents to be made up fresh and there are several manual steps involved including an overnight digestion. Multiple steps

introduce more errors, the Smart Digest available now from Thermo Fisher Scientific utilises a heat stable trypsin, which is active at 70°C, conditions at which other proteins will heat denature. The sample preparation can therefore be reduced to a simple dilution with digestion buffer followed by a 1 hour digestion at elevated temperature. Figure 1 shows the reproducibility of 3 separate digestions of Cetuximab.

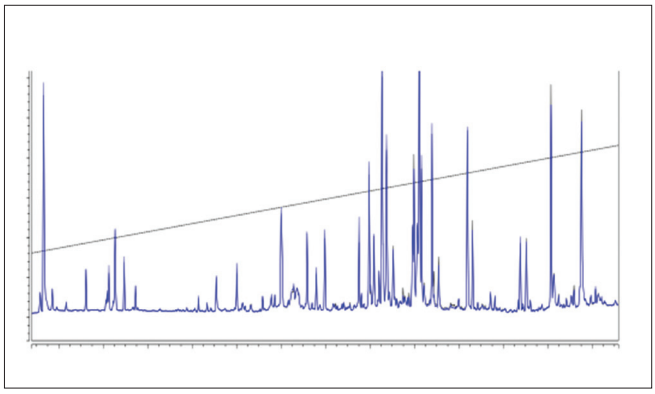


Figure 1: Overlay of 3 different digestions of Cetuximab produced using the Smart Digest. A Vanquish UHPLC was used with an Acclaim RSLC 120 C18 column 2 x 250mm. Eluent A is TFA 0.05%, Eluent B is acetonitrile/water 80/20, 0.04% TFA with a gradient o 4 to 50% B in 30 minutes at 80 with detection at 214 nm.

The column chosen for the analysis is a small particle size C18 column with a longer length of 250mm for higher resolution of the peptides. The retention time stability of the peptides averaged between 0.01 to 0.02, with an area precision between 2 to 8%, most of which were below 5%. The analysis including sample preparation can be completed before traditional methods have even begun the digestion. This is achieved with a much higher reproducibility and without the requirement of analysts highly trained in the art of trypsin digestions.

Charged Variant Analysis

The analysis of charged variants is a regulatory requirement for bio-therapeutic proteins. These large molecules can be subject to a variety of enzymatic post translational modifications during manufacture, such as glycosylation and lysine truncation. In addition to this, chemical modifications can occur during purification and storage such as oxidation or deamination. Ion exchange charged variant analysis is a high-resolution technique that has proved very useful in the analysis of such variants [4, 5, 7]. Common practice would involve individual ion exchange salt gradients for each MAb. The method development is time consuming until Genentech first published the use of pH gradients for this analysis instead of salt gradients [4]. The advantages included a global applicability of the method to any MAb, greatly simplifying the method development. Using shorter small particle size columns with linear gradients this can be improved further to bring this analysis into the domain of a true UHPLC application.

A difficulty with this technique is how to produce of a true linear pH gradient. Several buffers have to be employed to cover the whole of the pH range at concentrations so that the buffering capacities of each buffer match each other. The column itself will act as a buffer against any pH changes, so careful selection of a high resolution low capacity column is required. Many attempts at producing pH gradient buffer cocktails have been made and this can be seen in Figure 2.

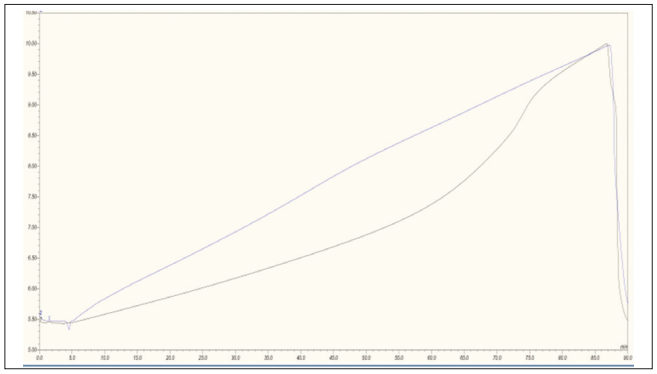


Figure 2: Monitored pH profiles using a BioRS UHPLC system equipped with a PCM-3000 pH and conductivity monitor. A MABPac SCX-10 RS, 4 x 250 mm column used with a programmed 80 minute linear gradient. Blue trace - Commercial CX-1 pH gradient buffers pH 5.6 to 10.2. Black trace - A published buffer cocktail [4] containing Tris, Imidazole and piperazine pH 5.6 to 10.2.

The difficulty in producing a linear pH gradient with buffer cocktails can be seen clearly from the black trace in Figure 2 where a clear curved gradient is produced in response to a programed linear gradient. There are many reasons why this is difficult to perform. Mixtures of monovalent and divalent buffer components make equilibration difficult, coupled with the different buffering capacities of buffer components in the cocktails. Charged buffers also interact with the charges on the resin giving a delay in the pH equilibration on column compared to that of the eluent itself. The commercially available CX-1 pH gradient buffers [blue trace], with the correct column produces the linear gradient that is programed into the UHPLC pump [7]. This enables method optimisation to be achieved easily and logically. Using UHPLC and a 5 µm ion exchange column the column length can be reduced to 5cm for faster run times. Figure 3 shows the type of fast method optimisation that can be achieved with any MAb for charged variant analysis. Starting with a linear gradient from pH 5.6 to 10.2 the antibody will elute somewhere along that global gradient depending on the PI range of the MAb. This can be achieved in 5 or 10 minutes with the shorter UHPLC column. From there it is easy to narrow down the pH range of the gradient to suit the individual MAb sample.

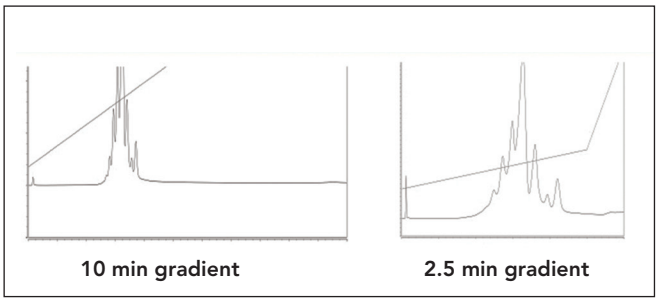


Figure 3: Optimisation of charged variant analysis using pH gradients. . A Vanquish UHPLC was used with a MABPac SCX-10 RS, 5µm analytical column 2 x 50mm. CX-1 pH gradient buffers were used, Eluent A is pH 5.6 Eluent B is pH 10.2. Panel A: Flow rate 0.45 ml/min, gradient 0 to 100%. Panel B: Flow rate 1ml/min gradient was 12 to 32% in 2.5 minutes.

The example in Figure 3 shows a typical method optimisation for Cetuximab with the initial 10 minute global gradient followed by the shorter 2.5 minute final analysis run time with the optimised pH range. The finalised run time is significantly reduced from a typical 60 minute run down to 2.5 minutes without any sacrifice in resolution.

In addition the method optimisation time has been reduced from weeks to a few minutes. Isoelectric focussing methods using capillary electrophoresis has also been used as a fast global method for charged variant analysis [9]. pH gradients are now faster with a global applicability that also allows the fractionation of any new variant peak found so that it can be positively identified with further analysis.

Hydrophobic interaction Chromatography [HIC]

The technique of using high salt concentrations to force hydrophobic interactions between a column and a protein is called hydrophobic interaction chromatography. The reduction of the salt concentration during the gradient allows elution of the protein. This technique allows the separation of proteins and protein variants through hydrophobicity in the native form. Reverse phase chromatography will also use hydrophobicity as the primary separation mode but due to the low pH, elevated temperature and solvent the proteins will be denatured. HIC allows separations in the native form and so can be used to monitor conformational changes in the protein as this will affect the number of hydrophobic sites accessible to the column matrix. Examples include separation of aggregates and fragments, allowing an additional mode of separation from SEC [10]. Post translational modifications such as oxidation can cause conformational changes allowing good separation on HIC columns [11, 12, 13]. A more recent application is the separation of ADC's with different drug ratios or DAR's. As each additional toxic drug is added to the parent MAb the resulting ADC has increased binding to the HIC column, making this column very useful in DAR analysis and also purification of ADC's with specific DAR ratios.

Traditionally HIC chromatography has a reputation for wide peak shapes and poor resolution. The new versions of HIC column have changed this considerably. The methods are again global to each MAb and ADC with one method having useful applicability to all proteins. The example shown in Figure 4 is of an ADC mimic that has the drug linked to the cysteine residues of the MAb. As there are several drug attachment points a number of isomers can be produced where the drug is attached to different places. These cannot be differentiated by mass spectrometry as they will be isobaric in nature and so chromatographic resolution is essential.

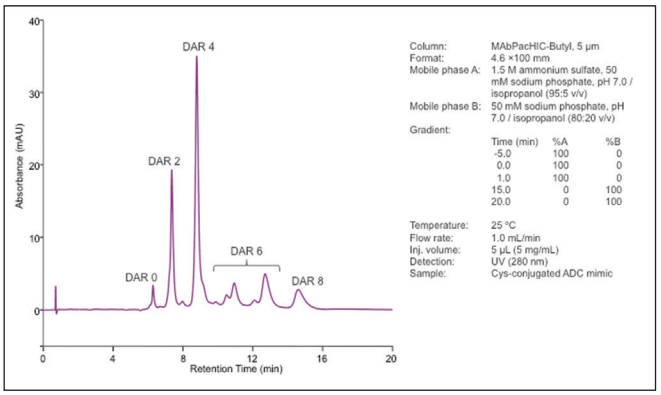


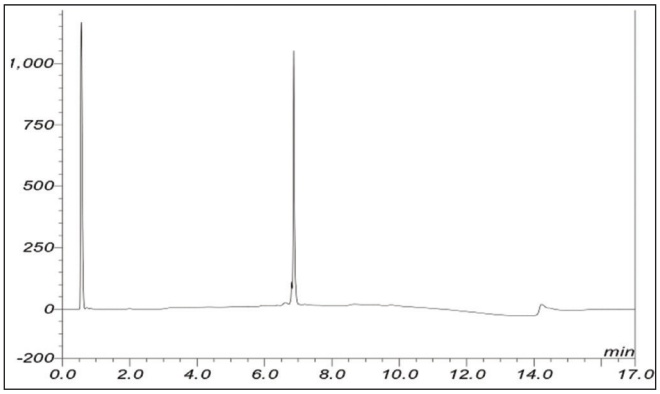
Figure 4: ADC analysis showing the DAR ratio of an ADC mimic. A MABPac HIC-Butyl analytical column 4 x 100mm was used. Eluent A is 1.5M ammonium sulphate, 50 mM sodium phosphate pH 7.0 / Isopropanol [95 / 5], Eluent B is 50mM sodium phosphate pH 7.0 / Isopropanol [80 / 20] with a gradient of 0 to 100% B in 15 minutes. The sample is a Cys-conjugated ADC mimic.

Figure 4 shows good separation of the individual DAR's with additional isomer separation. The ability to keep the protein in the native form during the analysis is essential as the attachment of the drug payloads at sites normally involved in disulphide bond formation can reduce the stability of the protein.

Reverse Phase Analysis

Another improvement in the analysis of large molecules has been achieved with intact protein chromatography on reverse phase. This analysis has been the domain of the silica C4 column for many years. The drawback in using silica resin technology for protein separations is the carryover effect from the silica column. With a protein as large as a MAb it is not uncommon to have carryover figures as high as 20%. The use of non-porous polymeric columns has alleviated that problem and there are now columns in this format capable of achieving higher resolution on large proteins that has only previously been associated with small molecule analysis [14]. A MABPac RP, 2 x 50mm analytical column was used in Figure 5. This is packed with a polystyrene divinylbenzene macro-porous resin. The carryover on this column from proteins is minimal, especially compared to silica resins. The peak width however is a low as 2 seconds at half height for a large MAb, bringing UHPLC resolution to large molecule analysis. Panel A shows the peak shape for a MAb rivalling that of a small molecule when used with one of the new biocompatible UHPLC systems. Panel B shows a 5 minute analysis of an ADC mimic giving a clear DAR ratio for this bio-therapeutic mimic. The attachment for this drug mimic is on the glycan structure already present in the FC domain of the parent MAb. The disulphide bonds are therefore still intact on the ADC preventing breakdown into heavy and light chains under the denaturing conditions used with this column.

A



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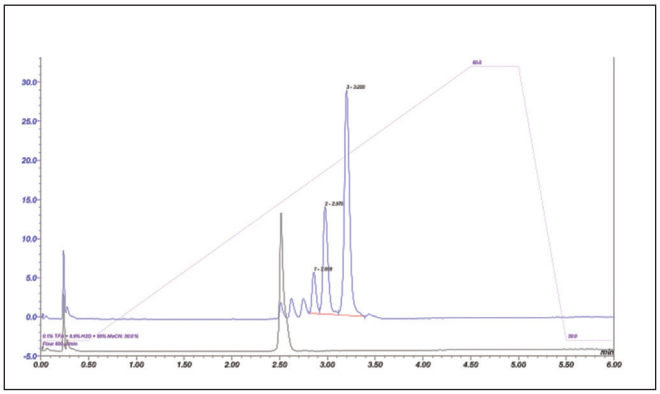

Figure 5 UHPLC Analysis of an intact MAb and ADC. A MABPac RP column was used. Column temperature 80°C, detection at 214nm. Eluent A is TFA 0.1%, Eluent B is Acetonitrile/water 90/10, 0.08% TFA . Panel A: Gradient from 0 to 100% B in 10 minutes, flow rate 0.3 ml/min, Sample is Rituximab. Panel B: Gradient from 10 to 60% B in 4.5 minutes, flow rate 0.6 ml/min, sample is an ADC mimic conjugated using a modified glycan attachment.

Glycan Analysis

This more commonly falls into a workflow which requires enzymatic release and separation of the glycans from the native MAb. This is followed by fluorescent labelling of the glycans and analysis by UHPLC or LCMS [15]. The sample preparation is not trivial and requires a lot of experience. This is essential for proteins with complicated very heterogeneous glycan structures. MAb's however, have relatively simple glycan patterns, which opens the door to a much simpler and quicker approach to this essential characterisation. The ultra-high resolution that can be achieved with Orbitrap mass spectrometry technology allows the characterisation of glycan structure on the intact MAb. The protein is introduced directly into the LCMS without any sample preparation [16, 17]. Any fraction from Ion exchange, SEC, or HIC containing high salt can be desalted on line using columns such as the new MABPac RP or polymeric monolith in under 5 minutes. In addition to a standard protein mass envelope the high resolution can see and identify the different glycan patterns within each individual charge state. This can be achieved with any orbitrap at relatively moderate resolution and gives the additional required information for average mass. Breaking the protein down to the smaller heavy and light chains by a simple reduction of the disulphide bonds enables accurate isotopic mass determination at higher resolution. This simple fast approach even allows for monitoring glycan structure during the fermentation process.

Instrument platforms

There is the obvious need for inert systems in bio-therapeutic analysis. A significant number of the column chemistries used here employ corrosive eluents which will eventually cause problems with steel systems. This can also have a detrimental effect on the columns due to metal contamination [18]. Some proteins and by no means all can bind to metals, in particular iron, on the column wall and frits resulting in distorted peak shapes, akin to the secondary type interactions that occur with bases and the residual acidified silonyl groups. Even if this is a small percentage the numbers of different drug candidates in the clinical pipeline only makes it a matter of time before a 'problem' protein is found. Many of the forms of chromatography that have been described for the analysis of proteins require the high buffer concentrations, which will cause corrosion in a stainless steel based fluidic system, due to the interaction of the salt and the iron. A few manufacturers have now introduced inert UHPLC systems to counter this problem with corrosion and so make the analysis platforms more stable, through the use of corrosion free metals such as titanium. Many of these chromatographic techniques mentioned previously for the analysis of proteins are orthogonal and so gives rise to the possibility of multidimensional workflows [19, 20] these new methodologies can be put together and implemented much more quickly. For example an affinity capture can be performed in 2 minutes with collection back into a cooled autosampler / fractionation module at high resolution. SEC analysis for aggregation can then be achieved in 4 minutes [21]. As the sample is still collected and present in the autosampler, a pH gradient variant analysis can then be performed in less than 5 minutes [Figure 3]. This means that complete characterisation workflows can now be undertaken on a single instrument at high throughput level. The software capabilities now available make this not only possible but relatively easy to perform. Many multidimensional systems have complicated plumbing requiring experienced analysts. The use of a fractionating autosampler and the ability to select different columns is a simple solution. Any peak from any column can be collected for further analysis without changing the plumbing configuration.

The benefits of increased resolution and selectivity using multidimensional workflows are alluring. However, the huge draw back has been the complexity of the system set up and the time it takes to perform. These high resolution systems now have the column chemistries to give the throughput required for complete characterisation in a short time.

Conclusions

Advances in instrument technology have been aligned with similar advances in column chemistries to bring large molecule analysis into the realms of UHPLC. It was a necessity to improve several chemistries alongside the instruments as the analysis of bio-therapeutics requires several FDA regulated characterisation protocols using an array of techniques which are quite different from those used in small molecule analysis.

Sample preparation for peptide mapping has been accepted as time consuming and difficult due to no other viable option. Smart Digestion not only removes this bottleneck but increases the reproducibility dramatically. This also removes the need for an experienced analyst, whose time can now be utilised more effectively.

The utilisation of high resolution chemistries and more efficient sample preparation accomplishes the goal of higher throughput without increasing the effort required. Ion exchange, reverse phase peptide mapping and intact protein analysis have been improved here to show highly selective resolution of different types of MAb variants to provide global methodologies. Many of these techniques however have eluent systems that are not compatible with MS detection. The initial positive identification of each variant peak from all of these chromatography methods must be achieved through mass spectrometry. Combinations of ion exchange, HIC and reverse phase HPLC/MS however can easily provide this with accurate fractionation of the UHPLC peaks followed by RP desalting.

The growth of the Bio-Pharmaceutical market has instigated true change in the capabilities of bio-therapeutic protein characterisation. Increased numbers of drug candidates are only one aspect as we also see new types of drugs emerging that require additional forms of analysis such as antibody drug complexes. This has increased the use and development of hydrophobic interaction chromatography where drug / antibody ratio analysis [DAR] has become a key application. This is a fast moving market that requires the analysts and the manufacturers to move quickly with it.

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