Analytics in Process Development - from Concentration to Characterisation

by Jim Martosella, Andrew Coffey and Phu Duong
Agilent Technologies, Inc.

Analytics are required at each stage of the purification process of a biopharmaceutical to determine the efficiency of the purification step. For example, a purification step designed to remove aggregates will be monitored using size exclusion chromatography (SEC) to quantify the efficiency of aggregate removal and to ensure that the required purity has been achieved before moving on to the next step. Whichever LC technique is employed, it must be robust. Where product purity is compromised then fractionation and characterisation can be instigated to determine corrective action and reduce the risk of losing valuable product.

Biopharmaceutical APIs and finished products undergo quality control testing to demonstrate their conformance to pre-determined specifications. Products produced by biotechnology are regulated by the same GMPs as classical pharmaceuticals but there are additional regulations or documents that relate to the methods used to determine their characteristics – identity, potency, purity, and impurity profile.[1-7]

The complexity of a biopharmaceutical dictates the use of more multiple and sophisticated analytical techniques. The molecules are much larger and are derived from living cells, and therefore have a complex pattern of product- and process-related impurities, which have implications for the purification and analysis of the finished product. For example, analysis of mAbs produced using bacterial fermentation can suffer interference from debris or proteins derived from the host cell itself. They can undergo complex post-translational modifications, individual molecules can aggregate, and mAbs have a specific three- or four-dimensional structure. Finally, mAbs are large molecules that can interact with the analytical system and be difficult to maintain in their native, functional state, so consideration must also be given to how they are handled and analysed.

Because pharma companies must monitor the purity and structural integrity of expensive monoclonal antibodies throughout the development process, they need accurate and sensitive methods that consume little sample, and provide rapid results.

Different analytical techniques are used at different stages, depending on the parameter to be assessed.

Physicochemical parameters

Primary structure, composition, and physicochemistry must be determined to assess identification and purity analysis of the biopharmaceutical. Primary structure information is derived from peptide mapping, C- and N-terminal sequencing and amino acid composition and sequence. Higher order structural information is obtained from spectroscopic methods including circular dichroism and NMR. Additional data, including molecular weight (size), isoforms, extinction coefficient, and electrophoretic pattern come from HPLC, MS or HPLC-MS for identification, homogeneity and purity analysis.

Biological activity

This is important as it determines the therapeutic effect of the drug, and provides information on functionality and proper protein folding. Methods may include in vivo bioassays. However, more reliable data may be obtained from in vitro assays developed using cell culture or other biochemical approaches, particularly where dose-response data are obtained. In each case, the assay must be carefully developed to provide the necessary potency data and to enable batch-to-batch comparisons to be made.[3,4]

Application	Technique	Notes
Capture and impurity removal	Affinity chromatography	A fast, single step method using affinity monoliths.
Primary structure analysis	UHPLC/HPLC reversed phase separations	RP separations require (or cause) denaturing of the protein to obtain detailed information on it.
Aggregation analysis, molecular size	Size exclusion separations	Protein aggregates are a major concern in drug product. They influence the final formulation and can cause immunogenic responses. They are quantified using SEC.
Chemical modification, charge variants	Ion-exchange separations	Protein analysis using ion-exchange requires buffered salt gradients or pH gradients.

Table 1. Liquid chromatography techniques for purifying and characterising monoclonal antibodies

Immunochemical properties

Antibody products require determination of immunological properties as these can be used to establish its identity, homogeneity or purity. Methods use binding assays to purified antigens and defined regions of antigens.

Quantity

The amount of purified protein is determined using UV spectroscopy. The absorbance at the maximum wavelength is measured and concentration calculated using empirical extinction coefficients.

Impurities

These can be product related or derived from the manufacturing process. Process-related impurities include the DNA and proteins of host cell, cell media and downstream processing aids. Product-related impurities are modifications such as isomerisation and oxidation, and post-translational modifications such as deamidation, truncation and aggregation. Impurities can affect the tertiary structure and antigen-binding properties of monoclonal antibodies.

In this article we examine the use of LC methods for the concentration and characterisation of biopharmaceuticals such as monoclonal antibodies. Table 1 shows the appropriate techniques, and Figure 1 is a diagram of a monoclonal antibody showing some of the features.

Concentrating mAbs and removing impurities

Impurities are product-related substances such as aggregates or deamidated, isomerised, and oxidised forms (charge heterogeneity) of the monoclonal antibody. They arise during cloning and production processes, and can affect the mAbs' tertiary structure and antigen-binding properties. In addition, changes in structure and form can alter the efficacy and toxicity of the drugs, for example, changes to the glycosylation site can critically affect the biological function of mAbs.

Monoclonal antibodies can be expressed from a variety of cell lines including mammalian, for example Chinese hamster ovary (CHO) cells, bacterial, such as the gut bacterium Escherichia coli, or yeast cell lines, for example Pichia pastoris. In all cases, the optimum time for harvesting is determined using affinity titer during the fermentation to measure the amount of mAb in the culture.

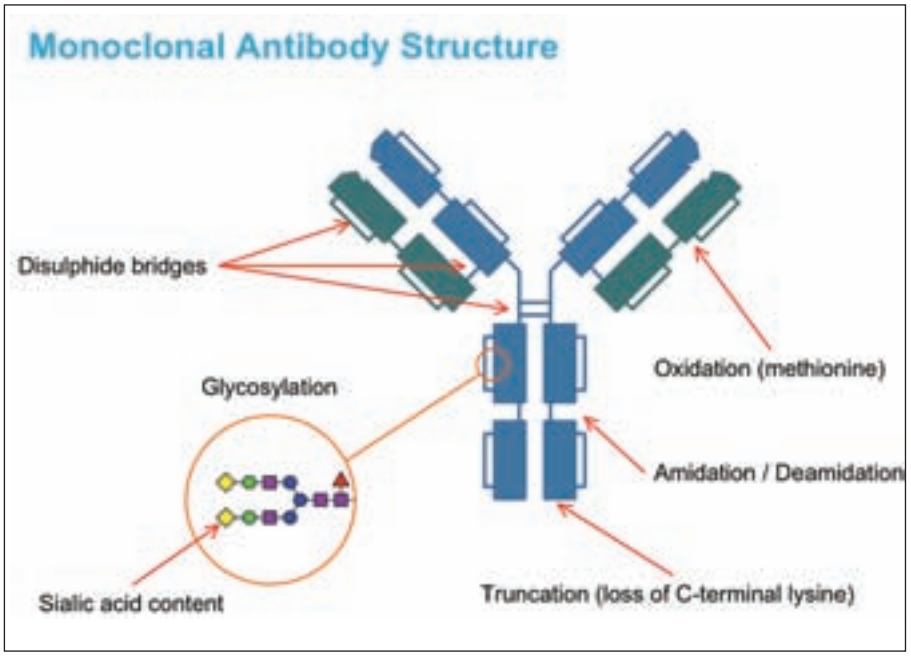

Figure 1. Structure of a monoclonal antibody.

The assay makes use of a monolithic protein A affinity chromatography. Bio-monolith Protein A columns are high-performance monolithic columns that offer all the advantages of a specially designed, continuous short polymeric bed. Their inherent features enable highly reproducible separation and quantification of immunoglobulin (IgG) from cell culture supernatants, at extremely high speeds.

Figure 2 shows that with optimised conditions, the method had very high affinity for IgG1. It prevents the non-specific binding of other proteins and allows only antibodies to be bound onto and eluted from the column. When the mixture of E. coli lysate and IgG1 was injected onto the column, only IgG1 was retained and eluted by 0.1M citric acid at 1.8 min retention time, whereas the bacterial lysate was not absorbed by the column and washed out at 0.1 min. The complete run took less than 4 minutes.

The linear response in Figure 3 demonstrates that Bio-monolith Protein A can quantitate different amounts of IgG1 when injected onto the column. Purified IgG1 was injected 10µg for the first separation, 5µg for the second run and 2.5µg for the third run. For 10µg, the mAU of UV 280 was 200, for 5µg was about 100 mAU and for 2.5µg was about 50 mAU (see Figure 2). The data indicated that the method delivered accurate and precise information on concentration to determine the best harvest time for monoclonal antibodies.

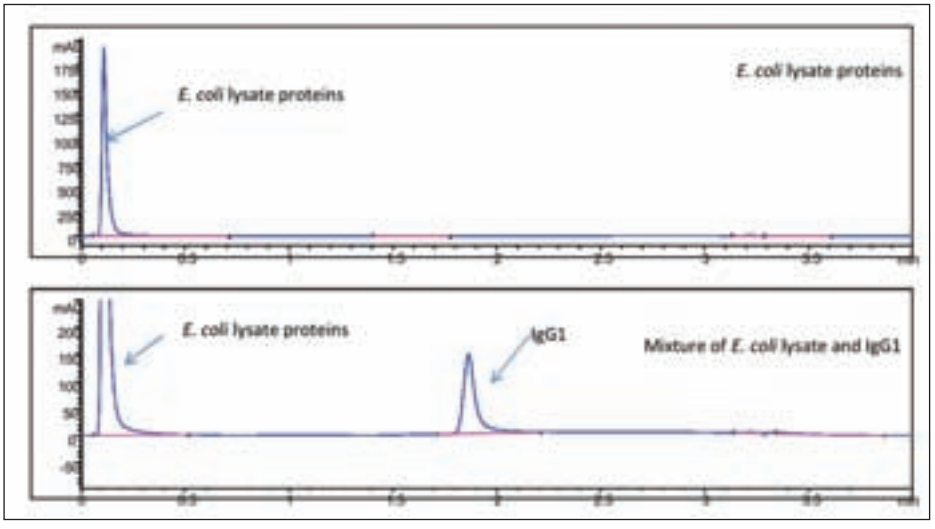

Figure 2. Specificity of Bio-monolith Protein A to IgG1. Experimental conditions: Column - Agilent Bio-monolith Protein A; Mobile phase: A (binding buffer) - Sodium Phosphate Buffer, 20mM, pH 8.0, B (eluting buffer) - Citric Acid, 0.1M, pH 2.8; Gradient: 0% B from 0-0.5 min (for washing), 100% B from 0.5- 1.5- 2 min (for eluting), 0%B from 2.1-4 min (for equilibrating); Sample: IgG1 (2.5mg/mL) and E. coli lysate (10mg/mL); Injection: 5µg; Flow rate: 1.0mL/min; Detection: UV at 280 DAD from Agilent Bio-inert 1260 LC system.

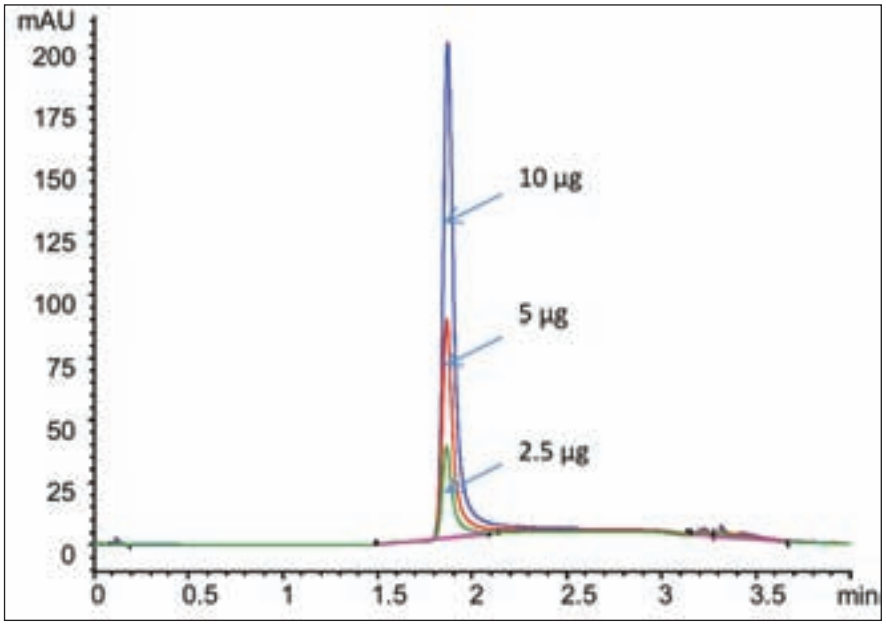

Figure 3. Different amounts of IgG1 quantitated on an Agilent Bio-monolith Protein A column. Experimental conditions as for Figure 1.

Primary structure analysis

The analysis of the primary structure, the sequence of amino acids, is very complicated and one that involves several steps. Enzymatically digesting the proteins and analysing the resulting peptides will generate a peptide fingerprint which, although it will not give the full primary sequence of peptides, will allow for comparisons between sample types to compare the amino acid composition. The complexity of the sample may well require that different digestive enzymes have to be used to get a better coverage of the structure. Analysis of the resultant amino acids can be done using conventional HPLC methods.

Small changes in primary structure alter secondary, tertiary, and quaternary structures of a protein. Reversed-phase UHPLC columns with 1.8 µm particles and 300Å pores are ideal for primary structure analysis to confirm protein identity, quantify post translational modifications, and fingerprint impurity profiles. With UHPLC at 1200 bar, faster separations can be achieved to improve productivity but with no compromise in data accuracy. The primary benefit of these columns, for protein separation, is increased resolution with shorter analysis times; resolution is critical in these separations due to the small differences in structure between the candidate protein and the "impurities".

Different phases, C18, C8, C3 and diphenyl, provide a range of selectivities for peptide mapping, small intact protein analysis, and larger recombinant protein and monoclonal antibody analysis. The C18 phase is the most hydrophobic and hence retentive of alkyl chains and is used for peptide mapping and analysis of small intact proteins. With decreasing alkyl chain length, the hydrophobicity decreases and the columns are used for larger proteins and more hydrophobic proteins. The diphenyl phase has the additional aromatic functionality with alternative selectivity based interaction with the aromatic amino acids in the protein primary structure (Table 2).

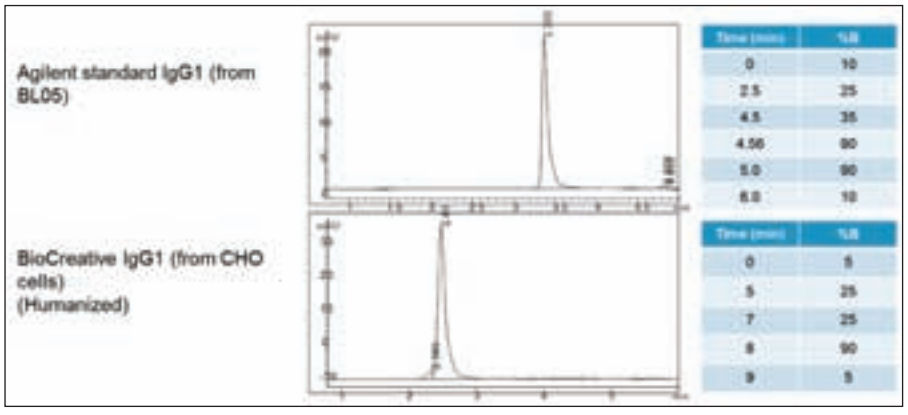

Figure 4: Fast separation of intact mAb from different cell lines. Experimental conditions: Column - Agilent ZORBAX RRHD 300SB-C3, 2.1 x 100 mm, 1.8 µm; Mobile phase: A - 0.1% TFA in Water, B - 70% Isopropyl Alcohol, 20% ACN, 9.9% Water + 0.1% TFA; Samples: mAb (IgG1) (1.0mg/mL) - BioCreative IgG1, Agilent Standard IgG1; Injection 2µL; Flow rate: 1.0mL/min; Temperature: 74°C; Detection: UV at 280nm.

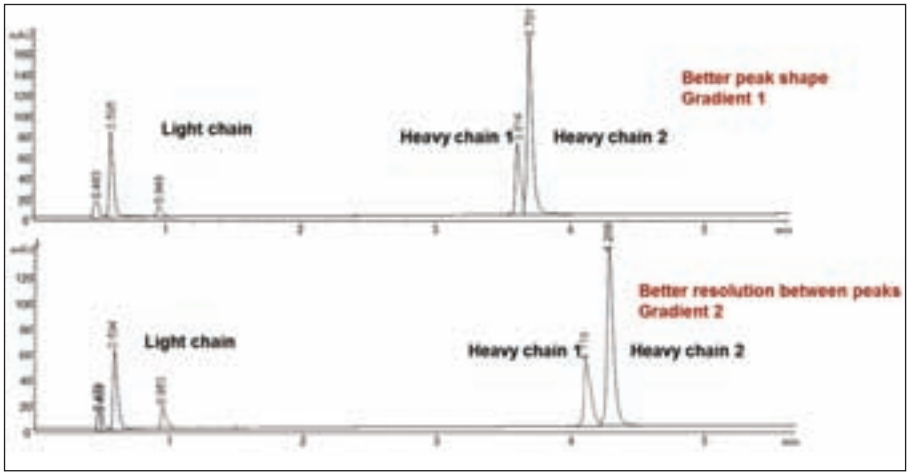

Figure 5: Fast separation of reduced and alkylated mAb. Experimental conditions: Column - Agilent ZORBAX RRHD 300Diphenyl, 2.1 x 100 mm, 1.8 µm; Mobile phase: A - 0.1% TFA in Water, B - 80% n-Propyl Alcohol, 10% ACN, 9.9% Water + 0.1% TFA; Samples: mAb (IgG1) (1.0mg/mL) - BioCreative IgG1, Agilent Standard IgG1; Injection 2µL; Flow rate: 0.5mL/min; Temperature: 74°C; Detection: UV at 280nm.

Ligand	Application
C18	Small intact proteins, peptide maps
C8	Intact proteins
C3	Larger or hydrophobic proteins, including mAbs
Diphenyl	Unique selectivity

Table 2. Ligands for reversed-phase chromatography of different biopharmaceuticals.

Figure 4 shows how the C3 phase determines monoclonal antibodies from different cell lines. The gradient was optimised for each antibody.

Figure 5 is an example of a separation of light and heavy chains of reduced and alkylated mAb using a diphenyl phase.

Aggregation analysis

Aggregates include dimers and higher multiples of the desired product. These are generally resolved and quantitated by using size exclusion chromatography or capillary electrophoresis. Aggregates, and larger particulate contaminants, are of particular concern because they are known to be a cause of adverse reactions, including potentially fatal anaphylactic shock.[6]

Size exclusion chromatography is an ideal tool for monitoring the amount of dimer or larger aggregates since the size of the molecule (in solution) is directly related to its retention time. Some other considerations when using SEC include:

- Peak height is related to detection mode – most frequently UV
- SEC does not concentrate sample and system dispersion must be kept to a minimum
- Non-specific (ionic or hydrophobic) interactions between the candidate molecule and the media must be avoided
- Mobile phase is modified to minimise non-specific interactions
- Mobile phase is modified to minimise non-specific interactions, including addition of organic modifier, n-propanol, or salt, sodium chloride or sulphate

In SEC the analytical conditions can increase or decrease aggregation levels. This can be assessed by performing a serial dilution to check for method-modified aggregation.

Size exclusion chromatography is a very simple technique in principle. The molecules should not interact with the sorbent in any way, but this means that there is none of the focusing effect resulting in an increase in concentration of the analyte observed with other forms of gradient elution chromatography, as it is not absorbed onto the column. The separation is conducted under isocratic elution conditions (no gradient needed), with relatively slow flow rates when compared to other chromatographic techniques.

Reversed phase HPLC is commonly carried out at 1.0mL/min on a column with an internal diameter of 4.6mm. This means the speed (linear velocity) is 360 cm/h. SEC is also commonly carried out at 1.0mL/min but the column diameter is 7.8mm, and so the linear velocity is 65% slower. Column lengths are typically 30cm and run times are often 20 minutes. However, the separation in Figure 6 was run on an Agilent Bio SEC-3 column, with a smaller 3µm particle that offers higher resolution separations at higher flow rates

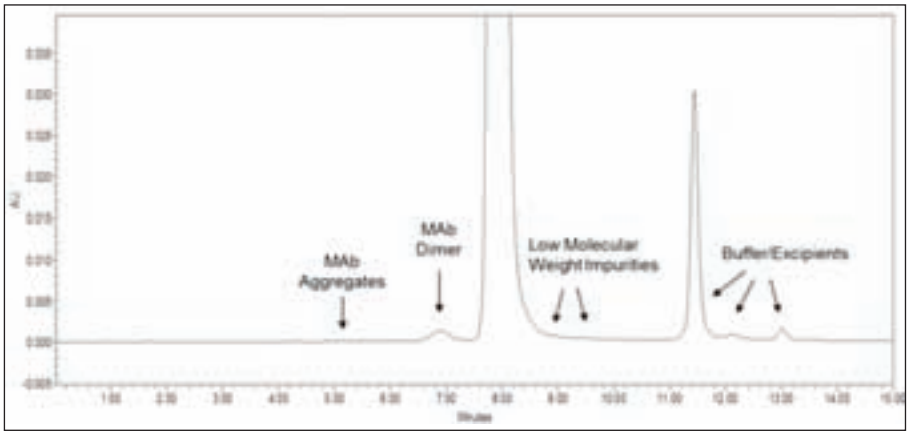

Figure 6: Monitoring aggregation and impurities of monoclonal antibodies using size exclusion chromatography. Experimental conditions: Column - Agilent Bio SEC-3 300Å, 7.8 x 300mm; Mobile phase: 150mM Phosphate, pH 7; Flow rate: 1.0mL/min; Temperature: Ambient: Sample: Monoclonal antibody (10µL, 5mg/mL).

and with shorter separation times. Note the baseline separation of the dimer and monomer.

Smaller particle size columns allow you to reduce the analysis time and improve throughput by permitting use of shorter column lengths without losing resolution, and permitting an increase in flow rate due to the reduction in peak widths, resulting in a greater resolution. Figure 7 is an example of the benefits of using a shorter column to markedly reduce analysis time.

Ion-exchange chromatography

Ion exchange separates on the basis of charge; with proteins this is based on accessible surface charge and the interaction between these charges and the stationary phase on the column. Retention is dependent on the strength and number of charges that interact with the column. Ion-exchange chromatography often keeps proteins in their natural state. It is the 3-D structure of the protein that determines which amino acid residues are on the exposed surface and are therefore available to interact with the stationary phase.

The best bonded phase to use will depend on the net charge of the protein. A cation exchange column should be used if the

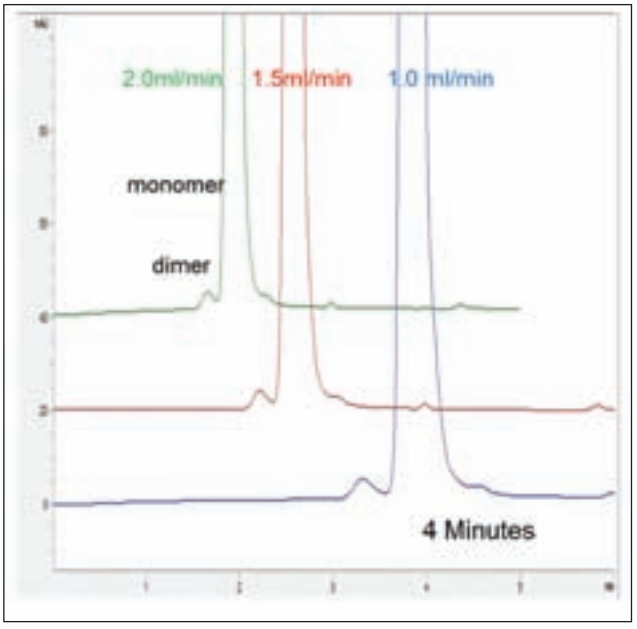

Figure 7: Resolution of mAb monomers and dimers in less than four minutes using size exclusion chromatography. Experimental conditions: Column - Agilent Bio SEC-3, 7.8 x150mm; Eluent: 150mM Sodium Phosphate + 100mM Sodium Sulphate Sample: mAb (2mg/mL); Injection: 5µL; Flow rate: 1.0, 1.5 and 2mL/min (56 bar , 75 bar, 105 bar); Detection: UV at 220nm.

Flow rate (mL/min)	Resolution monomer/dimer	Monomer efficiency	% Dimer
1.0	1.53	3510	0.64
1.5	1.43	2502	0.47.
2.0	1.13	1917	0.64

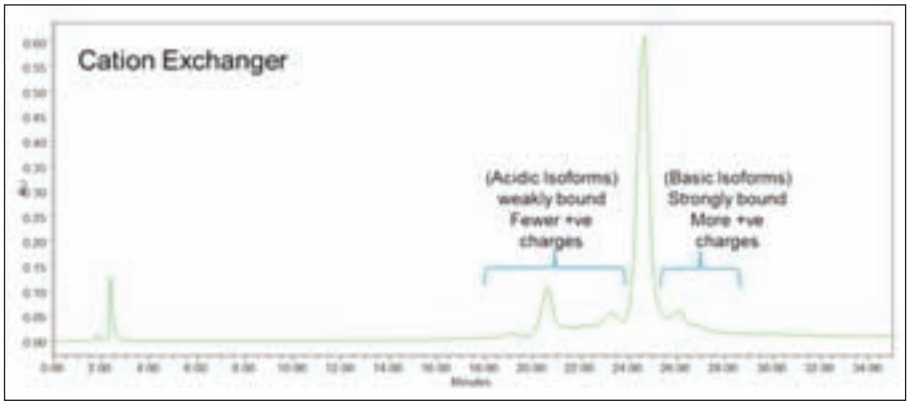

Figure 8. High resolution separation of acidic and basic charge variants using the Agilent BioMAb NP5 column. Experimental conditions: Column - Agilent BioMAb NP5, 4.6 x 250mm, 5µm, PEEK; Mobile Phase: A - Sodium Phosphate 10mM, pH 7.5, B - A + 100 mM Sodium Chloride, pH 7.5; Gradient:15 to 95% B in 60min; Sample: mAb, 5µL, 5mg/mL; Flow rate: 0.8mL/min.

mobile phase or sample pH is below the isoelectric point of the protein because this will provide a net positive charge. Alternatively, an anion exchange column is appropriate if the mobile phase or sample pH is above the isoelectric point, where more negative charge will be present. The one caution here is that isoelectric point is a guideline and not absolute. Like any other molecule, the interaction is dependent on the microenvironment of the interaction site. One example of this is where a molecule that surrounds itself with charges and so would not be able to interact with the column would result in an unexpected elution time.

In mAbs, charge variants (charge heterogeneity) are translational modifications, which can occur at sites of disulfide shuffling, deamidation or oxidation, glycosylation, or truncation (Figure 1). Acidic charge variant formation involves incomplete C-terminal processing of Lys and Asn, sialylation of terminal glycans, deamidation and isomerisation of Asn and Gln, Glu cyclization (= pyroglutamate), and adduct formation or control. Basic charge variant formation involves succinimide formation, oxidation of Met, Trp, Cys, and His, and disulfide-mediated conformational change.

The general rule for choosing a Bio IEX column is to select a SAX or WAX for acidic proteins, or a SCX or WCX for basic proteins. Consideration should also be given to the isoelectric point (pI) of the protein when choosing mobile phase pH. If the pH>pI, the protein will have a net negative charge, and if pH<pI, the protein will have a net positive charge. The pH of the starting buffer should be 0.5 to 1 pH unit from the pI; above pI for anion exchange and below pI for cation exchange.

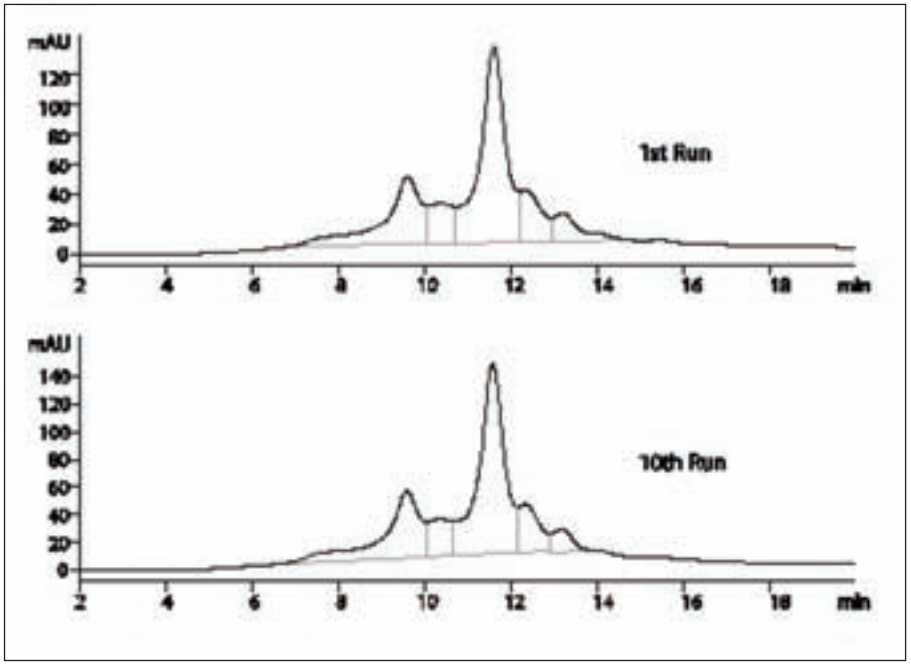

Figure 9. Consistent separation using an Agilent Bio MAb PEEK column in the metal-free flow path. Experimental conditions: Column: Agilent Bio MAb, 2.1 x 250mm, 5µm, PEEK; Mobile phase: A - Sodium Phosphate buffer, 20mM, pH 7.5, B - A + Sodium Chloride, 400mM; Gradient: 15 to 35%B from 0 to 30min; Sample: CHO-humanised mAb, 1mg/mL; Flow rate: 0.65mL/min; Injection: 2.5µL; Temperature: Ambient; Detector: UV at 220nm.

If the sample is sensitive to the presence of metal it is important to use a column that is made from PEEK rather than stainless steel, and to ensure that the rest of the flow path is also inert.

Figure 8 shows a charge isoform analysis of monoclonal antibodies using a bio-inert ion-exchange column.

Figure 9 shows the type of consistent ion-exchange mAb separation you can expect with the Agilent Bio MAb column.

Conclusions

Analytical LC techniques are utilised throughout the manufacturing process of a biopharmaceutical, from cell line stability and productivity to confirming product purity and freedom for contaminants at final product release. Affinity, size exclusion, ion-exchange, and reversed phase chromatography can all be employed, with each technique contributing complementary information on the target protein identity and impurity profile.

References

1. FDA/CBER, Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use, US Food and Drug Administration/ Center for Biologics Evaluation and Research (February 1997) http://www.fda.gov.

2. EMEA, ICH Topic Q 6 B: Specifications, Test Procedures and Acceptance Criteria for Biotechnological/Biological Products, European Medicines Agency (September 1999) http://www.emea.eu.int.

3. USP, <1032> Design and Development of Biological Assays, United States Pharmacopeial Convention (2010) http://www.usp.org.

4. N. Rieder, H. Gazzano-Santoro, M. Schenerman, R. Strause, C. Fuchs, A. Mire-Sluis, L.D. McLeod, BioProcess International, 8, 6 (2010) 33-42.

5. S. Kozlowski, BioPharm International, 20, 10 (2007).

6. S. Hermeling D.J. Crommelin, H. Schellekens, W. Jiskoot, Pharmaceutical Research, 21, 6 (2004) 897-903.

7. A.K. Pavlou, M.J. Belsey, European Journal Pharmaceutics and Biopharmaceutics, 59, 3 (2005) 389-396.