Analysis of Biopharmaceuticals to Conform to ICHQ6B

David Neville, Technical Specialist and Andrew Kenyon, Technical Specialist

Reading Scientific Services, Reading Science Centre, Whiteknights Campus,
Pepper Lane, Reading, Berkshire, RG6 6LA
Tel: +44 (0)118 918 4000 Email: enquiries@rssl.com Web: www.rssl.com

Introduction

Worldwide, the pharmaceuticals market is anticipated to grow from more than USD 782 billion in 2011 to approach a value of just over USD 971 billion by the end of 2016, registering a CAGR of over 24%[(1)]. In 2010 the average medicines expenditure per person within the UK was £271[(2)], and this is expected to increase with the ageing population. A significant and increasing proportion of these sales are protein-based biotherapeutics or biomolecules. Currently, these account for 19% of the total market, and are growing at twice the rate of traditional small molecule pharmaceuticals. It is predicted that close to 50% of the top 100 pharmaceutical products will be biomolecules by 2016[(3)]. By far the largest segment of the biopharmaceutical market is the monoclonal antibody (MAb) with an estimated share of 25.6%, which corresponds to USD 51.1 billion[(4)].

It is over 25 years since the first biopharmaceutical – Humulin - made from recombinant DNA (r-DNA) was approved for human use. To date over 300 biopharmaceuticals have been approved worldwide. Many of these blockbuster drugs will go off patent over the next few years. This paves the way for the production of biosimilars - a 'generic' biopharmaceutical similar but not identical to the originator product. To gain regulatory approval, significant analytical data is required to confirm that the product has the same quality, safety and efficacy.

This paper highlights some of the analytical techniques that are used when setting specifications for biomolecules to meet the guidelines set out in the document 'Specifications: Test Procedures and Acceptance Criteria For Biotechnological/Biological Products (ICHQ6B)'[(5)]. This document states 'A physicochemical characterisation program will generally include a determination of the composition, physical properties, and primary structure of the desired product. In some cases, information regarding higher-order structure of the desired product (the fidelity of which is generally inferred by its biological activity) may be obtained by appropriate physicochemical methodologies.' The complexity of proteins means that a number of techniques must be used in order to characterise them fully, and the primary, secondary and tertiary structure, as well as the physiochemical properties of the protein, should be assessed. Therefore, the specifications should focus on those molecular and biological characteristics found to be useful in ensuring the safety and efficacy of the product.

The manufacture of biopharmaceuticals can be complex due to the fact that they are produced within living cells. These include bacterial, yeast, plant and eukaryotic cells. Biopharmaceuticals may induce an immune response within the patient and in some severe cases can be potentially lethal[(6)]. Therefore, the steps required to purify the final product are complex, as is the testing regime. Processes must be tightly controlled to ensure a consistent product that meets the submitted specifications for the biomolecule. With this comes the additional challenge of the immunogenic potential and toxicity of any process-related contaminants. The choice of analytical techniques used must cover both the quality and consistency of the final product and the identification of any process-related contaminants. These must be determined throughout the discovery phase of product development, and in batch release monitoring of final product.

Electrophoresis

The issue of protein purity may be addressed by molecular weight-based separation techniques. The separation may be performed using high-performance liquid chromatography (HPLC), or more commonly by using polyacrylamide gel electrophoresis (PAGE). Consistency in analysis may be achieved by using capillary microfluidic chip gel electrophoresis systems, such as the BioAnalyzer 2100 system from Agilent or the LabChip Systems from Perkin-Elmer. These methods only require a fraction of sample compared to PAGE analysis; it is possible to detect protein at concentrations of 4 pg. The costs involved in developing biopharmaceuticals are very high, the smaller the sample used during analysis the more advantageous. In addition these systems are 21 CFR Part 11 compliant, essential for GMP analysis. By careful choice of sample preparation, biomolecule relative molecular weight characteristics may be determined under native and reducing conditions. These analyses can determine native protein relative molecular weights, the presence of aggregates and truncated products. The presence of inter- or intra-chain disulphide bonds and their effect on relative molecular weights and the relative molecular weights of individual protein chains can be determined under reducing conditions.

Figure 1 demonstrates the results of a typical analysis, pre- and post-enzymatic N-linked oligosaccharide removal, of a monoclonal antibody using a BioAnalyzer system. Only the heavy and light chain bands, and no other protein contaminants, can be seen in the antibody alone sample. This shows that the monoclonal is essentially pure, with no other obvious protein contaminants being present. It also shows that only the antibody heavy chain has a molecular weight shift post- N-linked oligosaccharide removal. This identifies that only the heavy chain contains N-linked oligosaccharides and that all the N-linked oligosaccharides are removed following treatment with PNGase F.

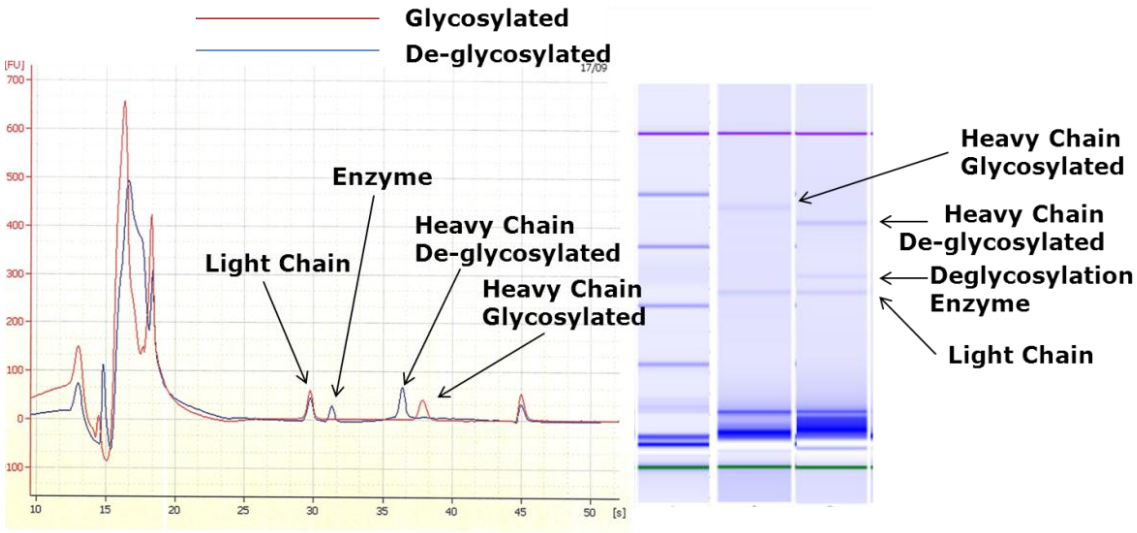

Figure 1: SDS-PAGE of an antibody pre- and post-removal of N-linked oligosaccharides using peptide-N-glycosidase F (PNGase F)

Mass Spectrometry

Confirmation of primary structure can be addressed, in part, by the use of high resolution mass spectrometry. Experiments may be performed on either whole proteins or on proteins digested with proteases to generate peptides unique to the protein being analysed. By the latter, process peptide masses, sequences, disulphide bond patterns and other post-translational modifications (glycosylation, phosphorylation etc.) are determined. This is necessary to ensure protein specifications are met for batch release, and any variance found is within defined limits.

Mass spectrometric analysis of whole proteins offers its own specific challenges, mainly due to their size. At a basic level, mass spectrometry determines the mass of a protein (often to ≤1 Da), and so provides a quick and easy way to accurately determine the extent of protein modifications and isoforms. With antibody-based drug products being in the region of 150 kDa, intact mass analysis can prove challenging as many mass spectrometers will struggle to see a protein that large. With electrospray instruments, each protein form will produce a charge state envelope consisting of multiple peaks, representing the same protein with different charge states. When looking at an intact antibody, charge states of up to 70 are required to bring the ions into the typical mass range of the electrospray instrument. The charge state envelope is then deconvoluted manually or by use of appropriate software.

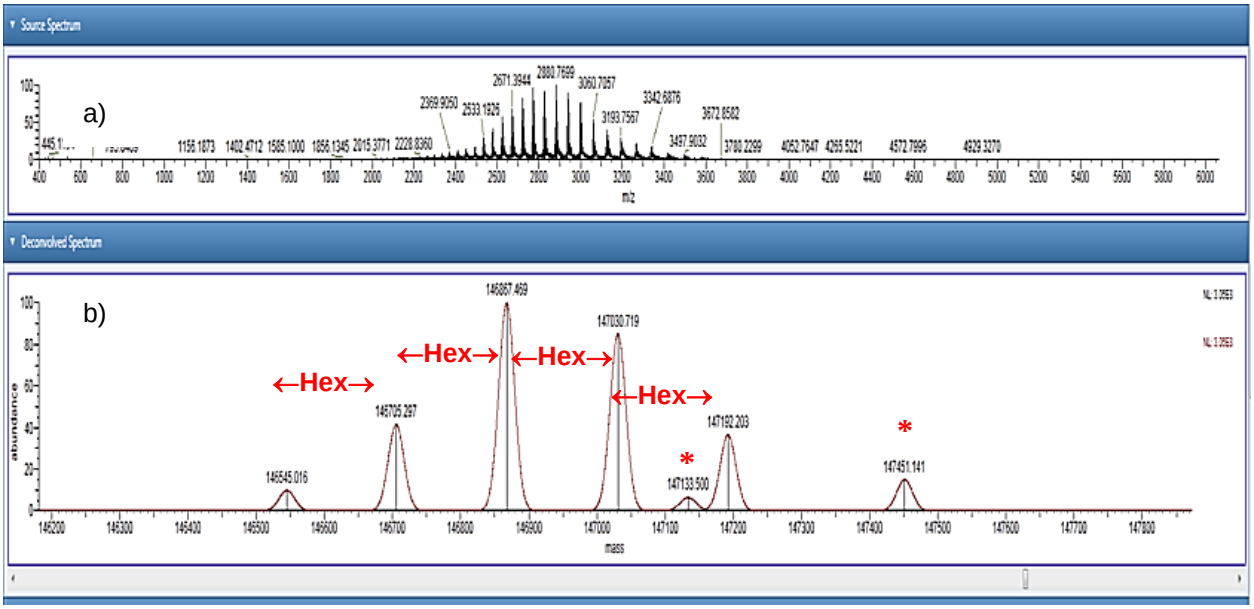

Figure 2: Intact mass analysis of a human monoclonal antibody

a) Raw mass spectrum showing the charge state envelope
b) Deconvoluted spectrum showing seven protein isoforms in the mass region of 147 kDa
*Unknown isoform modifications

Figure 2 shows an intact mass analysis of a purified human monoclonal antibody using a Q-Exactive (Quadrupole-Orbitrap hybrid) mass spectrometer. The deconvoluted spectrum produces seven isoforms. Five of these isoforms can be seen to vary by a hexose (mass of 162 Da) unit. The modifications that result in two of the isoforms seen are not immediately obvious, but could be due to, for example, amino acid substitutions or N-terminal modification. Peptide mapping could be used to identify these unknown modifications. Once a more complete characterisation has been done as part of the initial stages of

regulatory approval, intact mass analysis of the antibody product provides a fast and easy method for routine quality control. This also highlights that mass spectrometry can give an early indication of the purity of the expressed and purified protein. Reduction and deglycosylation by PNGase F (an enzyme that cleaves only N-linked glycans) of the intact antibody can yield further information regarding the heavy and light chain masses and confirmation that observed glycans were indeed of the N-linked designation.

N-linked Glycosylation

Oligosaccharide analysis may involve several varied and complex methods such as high performance liquid chromatography, mass spectrometry, lectin-based analysis, enzyme digestions, chemical methods etc.

In most cases, it is first necessary to release the oligosaccharides from the protein. Enzymatic release is the preferred method, using peptide N: glycosidase F (PNGase F) which removes all N-linked oligosaccharides from mammalian proteins. Most biopharmaceuticals are currently produced using mammalian cell lines, meaning PNGase F should be appropriate in all cases. The oligosaccharides are further purified, prior to subsequent analysis, to remove any salt, detergent and protein contamination.

By linking fluorescent molecules to the core-GlcNAc residue (formerly attached to the protein backbone Asn residue of the oligosaccharide) it is possible to increase the sensitivity of the technique. A number of different fluorescent reporter groups are reported in the literature[7,8] and these influence sensitivity, choice of column, efficiency of separation and mass spectrometric analysis. By use of volatile HPLC solvents and the methods of separation outlined below, it is possible to interface the HPLC systems to mass spectrometers to increase the amount of information obtained. This information includes the mass of the individual oligosaccharides and the potential structural sequence of the oligosaccharides.

The most popular technique used to analyse fluorescently derivatised oligosaccharides is hydrophilic interaction liquid chromatography (HILIC). This is a form of HPLC routinely performed using an amine- or amide-derivatised column matrices on HPLC systems with fluorescence detection. The retention times of the oligosaccharides are usually compared to that of an external standard, and expressed in terms of glucose unit (GU) values. Recently, it has become possible to perform the same analysis using an internal standard method that provides more accurate GU values. The GU values obtained with different column matrices may be used to generate oligosaccharide retention chromatography databases. Figure 3 shows the separation obtained and the assigned oligosaccharide structures obtained from oligosaccharides released and labelled from a monoclonal antibody. With the recent advent of ultra-performance liquid chromatography (UPLC), or ultra-high-pressure liquid chromatography (UHPLC), the separation time has been decreased and peak resolution has dramatically increased when using HILIC-based separations.

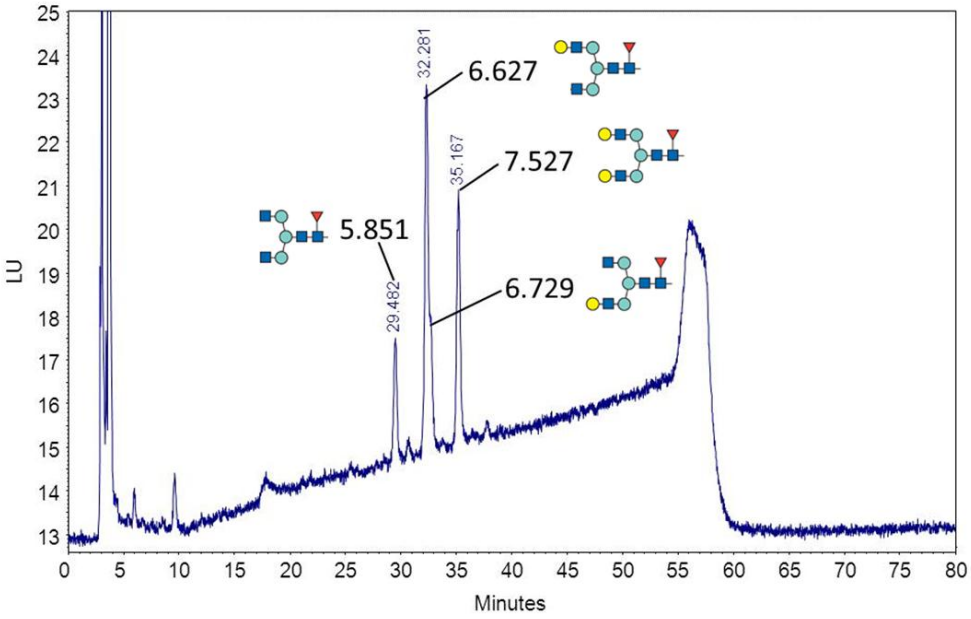

Figure 3: HILIC separation of anthranilic acid (2AA)-labelled oligosaccharides released from a monoclonal antibody

Analysis using HILIC may also be performed in combination with weak anion-exchange (WAX), strong anion-exchange (SAX), porous graphitized carbon (PGC) or reverse-phase (RP) separations. This combination of techniques greatly increases the ease of analysis and the number of different structures that can be observed.

It is possible to combine different chromatographies, hydrophilic interaction and anion-exchange, using a single column or a tandem column arrangement (so-called mixed-mode chromatography). It is also now possible to obtain GU values following hydrophilic interaction anion-exchange chromatography (HIAX) using different commercially-available column matrices. This offers the benefit of distinct separation of neutral from charged, sialic acid-containing oligosaccharides but using volatile HPLC solvents. Additionally, HIAX may be coupled to mass spectrometry to obtain oligosaccharide mass and sequence information.

Quantitative PCR (qPCR)

Residual host cell DNA and recombinant DNA (r-DNA) that has been transfected into the host cell is perceived as a principal risk associated specifically with continuous cell line derived products. It is not the DNA *per se* that is the concern, but the actual coding sequence that may have a potentially tumourigenic effect in humans. The regulatory authorities have therefore set maximum levels of residual host cell DNA per dose within biopharmaceuticals. The World Health Organization (WHO)[9] and the European Medicines Agency have set levels of 10 ng per dose, and the Food and Drug Administration (FDA) a maximum of 100 pg per dose. There are currently no set guidelines to the length of the DNA fragments; however, it is generally accepted that 90% should be less than 500 base pairs in length. DNA is removed from biopharmaceutical products by sequential purification steps and this is a crucial stage in the production of the final product.

The predominant method that has distinct advantages for the detection of residual DNA is by quantitative PCR (qPCR). The analysis is quantitative and relatively rapid taking around two hours. The method involves the expediential amplification of a short DNA sequence, approximately 100 - 200bp in length, by a series of polymerisation cycles. The amplification of the DNA sequence is measured in real time at each progressive cycle using a sequence specific fluorescent probe. The increase in the amplification of the DNA is directly proportional to the intensity of fluorescence. A sample that contains a lower initial DNA concentration will require more polymerase cycles to increase fluorescence above background level (ct) than that of a sample with a higher initial concentration (Fig 4). From this data a standard curve can be generated to calculate the concentrations of unknown samples (Fig 5). The limit of quantification (LOQ) can be in the region of 30 fg well below the regulatory guidelines.

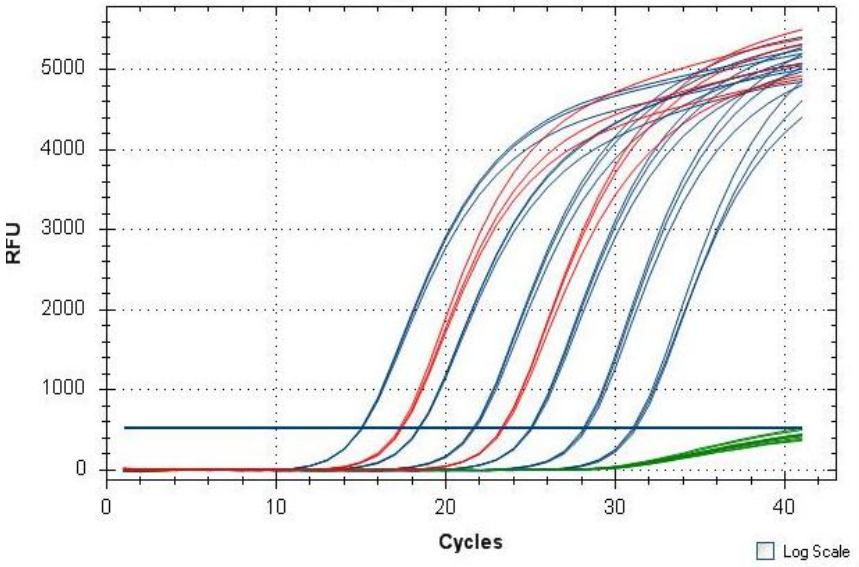

Figure 4. Amplification data

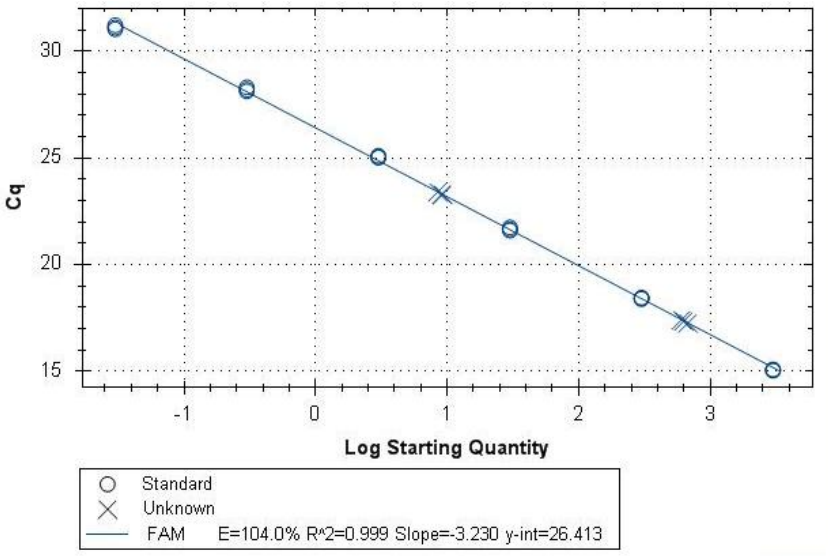

Figure 5. Standard curve

A second method for the quantification of DNA is using PicoGreen™ fluorescent dye. The method utilises the increased fluorescent intensity that is observed when PicoGreen™ binds to double stranded DNA (dsDNA). The intensity of the signal is directly proportional to the concentration of DNA within a sample. This can be compared to a standard curve generated from standards of known concentration. This method has a LOQ of 20 pg/ml and can detect DNA fragments as low as 50 base pairs (bp). The main disadvantage of this type of analysis is that it is non-specific, any contamination of DNA from another source would result in false positives.

A final method utilises DNA binding proteins to quantify single stranded DNA (ssDNA), commonly marketed as a DNA threshold assay. The method involves two sequence independent binding proteins. The first a biotinylated single stranded binding protein, the second an anti-ssDNA antibody conjugated to the enzyme urease. The sample is incubated with both proteins and streptavidin to form a protein DNA complex. This is filtered using a biotinylated membrane capturing and concentrating the DNA complex on the membrane utilising the strong affinity of streptavidin binding to biotin. The detection involves the addition of urea that is hydrolysed by the urease producing a change in pH that is proportional to the DNA concentration. This system has an LOQ of 2 pg however; the DNA fragments need to be greater than 500 bp and it is a much longer process than the previous two methods.

ELISA

One of the main issues with the production and development of a biopharmaceutical is that of immunogenicity. The two most common causes of an immunogenic reaction are the biopharmaceutical itself or process-related contaminants, including host cell proteins (HCPs) and proteins from the purification stage. Many purification steps may be required and these steps themselves have the potential to co-purify and concentrate these contaminants along with the biopharmaceutical. All biopharmaceuticals must therefore be purified to reduce these contaminants in order to minimise the incidence of immunogenicity against a biopharmaceutical or its trace contaminants[10]. Acceptable levels of these contaminants have not been set by the regulatory authorities and are determined on a case-by-case basis. This is due to many variables including dose, dosage frequency and drug delivery. However, it is generally considered that host cell protein levels should be less than 10 µg/ml[11]. The lack of purification during development has the potential for the product to fail in the early stages of clinical trials.

The most common detection system for host cell protein contamination is by enzyme-linked immunosorbent assay (ELISA). There are a number of commercially available ELISA kits on the market supplied by a number of companies against many cell lines including Chinese hamster ovary cells (CHO) and *E.coli*[12]. Most of these ELISA kit are “sandwich” based ELISA’s involving two antigen specific antibodies one bound to a microtitre plate to capture the antigen and a secondary antigen specific antibody conjugated with an enzyme the forms the antigen “sandwich”. The secondary antibody facilitates a reaction with a substrate colour change. The amount the substrate develops is directly proportional to the antigen concentration. The LOQ of these kits maybe in the region of 1 ng/ml, depending on the kit specifications. Commercially available ELISA kits maybe used to determine HCP levels within biopharmaceuticals in the early stages of development. However the regulatory authorities may request that a process specific ELISA kit is developed for each individual product before it enters phase III studies.

The process of developing a process specific HCP assay is long and complex, this must be started early in the development process to ensure a robust assay is in place prior to phase III trials. This development process my take in excess of 12 months, the rate limiting step is raising antigen specific antibodies will high sensitivity and low cross-reactivity. The initial step of the process is the production of antigen (HCP’s) from a mock fermentation process. This involves the fermentation of the cell line in the absence of the gene sequence encoding the biopharmaceutical[13]. The media is purified using the identical procedure used during product manufacture. This will contain antigens without the biopharmaceutical product that may be

used to generate polyclonal antibodies. As previously mentioned there are a number of purification steps within production, each with a different HCP profile, it must be considered from which of the purification steps the polyclonal antibodies are generated for a representative assay for the analysis of a final product. To raise polyclonal antibodies with a broad range of affinity to the HCP it may necessary to adopt a number of immunisation schemes. The immune response should be stimulated against strong and major antigens as well as the weaker less predominant ones. One method is to mix antibodies from previous bleeds with the immunogen and re-immunise. This blocks the major antigens resulting in a stronger immunogenic response to the weaker antigens, passive immunisation[14]. Secondly or a combination of, antibodies from previous bleeds are used to eliminate dominant antigens from the immunogen by immunoaffinity chromatography. This enriches the weaker antigens that may be used in the next immunisation schedule, cascade immunisation[15].

The raised polyclonal antibodies are purified by affinity chromatography and quantified following gel electrophoresis and western blot analyses. The aim of the process is to raise antibodies with the maximum detection of the HCP contaminants[16]. The antibody coverage can be analysed using 2D electrophoresis. The coverage of 80% in the pI range of 4 to 7 and a molecular weight between 10 and 120 kDa is achievable. There are a number of methods for using these antibodies to develop an ELISA including direct, indirect, competition and “sandwich”, the choice of which will be dependent on the antibodies and antigen. The main considerations for the development are to ensure a sufficient LOQ that is able to satisfy the regulatory authorities and that the final antibody selection has low cross-reactivity. Both the antigen and antibodies need to be stored to be used as reference materials for future assays.

Stability

Stability testing is essential for all pharmaceuticals to demonstrate how the quality of the product is affected with time under a number of conditions including temperature, humidity and light[17]. The physiochemical characteristics can be affected over time and storage conditions, these may include, quaternary, tertiary and secondary structures affecting the safety, quality and efficacy of the product[18]. Analytical data from these studies can be used to recommend the shelf-life and storage conditions of the product. There is a finite balance of formulation conditions needed to ensure product stability including pH and osmolality. Many biopharmaceuticals are lyophilised prior to storage, however even under these conditions degradation may occur[19]. Additionally degradation of biologics over time and with storage conditions means that stability studies need to be performed to ensure the drug is still effective and does change into something potentially immunogenic.

Conclusion

The growth of the biopharmaceutical industry has continued to increase since the production of the first recombinant human insulin “Humulin” in 1982. In the next few years the number of approved biopharmaceuticals is expected to exceed that of their small molecule counterparts. This paper emphasises the array and complexity of the analyses that are required to ensure that the final product will satisfy the lowest immunogenic potential for the patient. Continued pharmacovigilance is important as biopharmaceutical product contraindication(s) incidents may differ depending on individual patient characteristics [20] and the potential of batch-to-batch variation.

The patent expiry of a number of early biopharmaceuticals has spawned the race for the next generation of ‘biosimilars’ or ‘follow-on-biologics’ to be developed and produced. Although early research and development costs may be saved, relative to that of the originator molecule, extensive analytical testing is still required to demonstrate that the biosimilar’s physical and chemical characteristics closely match that of the originator reference product. Adherence to the regulatory analytical guidelines set out in the ICHQ6B

publication need to be demonstrated to ensure market approval, and release of product. New guidelines are currently under review by The Federal Drug Administration (FDA) could result in biosimilars being approved for use in the US. The extensive analytical portfolio, therefore, ensures the quality, safety and efficacy of biopharmaceutical products.

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