Conducting Non-clinical Studies with Protein Biologics: Considerations in Test Article Characterisation and Method Development for DoseFormulation Analysis

Introduction

Historically, the majority of non-clinical studies conducted under Good Laboratory Practices (GLP) regulations[i] involve synthetic small molecule chemical entities. As a result, general GLP practices from testing facilities, including those for contract research organisations (CROs), have been developed from experiences working with this type of molecule. With the increase in the number of protein biologics in development, including monoclonal antibodies, the proportion of GLP studies conducted for protein test articles has correspondingly increased. Therefore, current GLP practices may need to be adjusted to accommodate the physiochemical characteristics of protein test articles, such as a different interpretation of test article characterisation requirements. In terms of method development for non-clinical dose formulation analysis, analytical techniques specific to protein biologics will need to be used.[ii] This article describes the current best practices applied in the author's organisation for test article characterisation and analytical method development of protein biologics for non-clinical dose formulations. For the purpose of this article, a protein biologics test article refers to a protein formulated in a buffer to be used for preparing dosing formulations for non-clinical studies.

Test Article Characterisation

According to Section 58.105(a) of the GLP regulations, "The identity, strength, purity, and composition or other characteristics which will appropriately define the test or control article, shall be determined for each batch and shall be documented."[i] For non-clinical testing at a CRO, the sponsor must provide this information in the form of a certificate of analysis, or a statement of testing for the particular lot of test article to be used in non-clinical testing. The certificate of analysis must be maintained as part of the GLP study records. A sample certificate of analysis for a monoclonal antibody protein test article is shown in Figure 1. This example certificate of analysis illustrates common characterisation information generally

Figure 1: Sample Certificate of Analysis for a Monoclonal Antibody Test Article

CERTIFICATE OF ANALYSIS

Date	01 Oct 2012
Identity	MAB-4321
Formulation Buffer	10 mM Histidine/Histidine-HCl, 150 mM NaCl, 1% (w/w) trehalose, 0.01% (v/v) Tween 80, pH 6.0
Lot Number	ABC123
Storage	-50°C to -90°C
Date of Manufacturing	01 Sep 2012
Retest Date	01 Sep 2013

SUMMARY OF ANALYSIS RESULTS

Method	Parameter Measured	Specification	Results
Appearance	Appearance	Clear colourless solution	Clear colourless solution
pH	pH	pH 5.5-6.5	pH 6.0
UV (A280)	Strength/protein content	8.5-11.5 mg/mL	10.1 mg/mL
SDS-PAGE (non-reduced)	Identity and purity of intact IgG	Comparable to reference standard	Comparable to reference standard
SDS-PAGE (reduced)	Purity of light and heavy chain of IgG	Comparable to reference standard	Comparable to reference standard
SEC-HPLC	% monomer	≥ 95% monomer	96.5% monomer
cIEF	Isoelectric Focusing Profile	Comparable to reference standard	Comparable to reference standard

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supplied by the sponsor for a test article. Depending on the extent of characterisation work that has already been conducted, there may be additional tests and results listed beyond the examples provided.

In Figure 1, the test article is uniquely identified as MAB-4321. The method used for its identification is a non-reduced SDS-PAGE method. The methods of non-reduced and reduced SDS-PAGE, size-exclusion chromatography (SEC-HPLC), and capillary isoelectric focusing (cIEF) are used to determine the purity of the bulk test article. A UV (A280) method is used for the strength or concentration measurement of the protein in the bulk material[ii] As a protein test article is generally supplied formulated, the formulation buffer listing the concentrations of the various components in the buffer is also given. Therefore, for this example, the certificate of analysis provided demonstrates how the GLP requirements of identity, strength, purity, and composition can be met for the test article.

Section 58.105 (b) of the GLP requirement also states, "The stability of each test or control article shall be determined by the testing facility or by the Sponsor either (1) before study initiation, or (2) concomitantly according to written standard operating procedures, which provide for periodic analysis of each batch."[i] In the example shown in Figure 1, the bulk test article is to be stored at -50°C to -90°C, with a re-test date of one year from manufacture, indicating the stability of the material at this storage condition for a period of one year. Additionally, a statement from the sponsor providing information such as refrigerated stability and freeze/thaw stability can constitute part of the stability requirement, in addition to assisting in the planning and conduct of the non-clinical study. For all the information provided to the CRO regarding test article characterisation and stability, it is the sponsor's responsibility to maintain relevant documents and results to support the information provided.

Analytical Method Development for Dose Formulations Analysis of Proteins

The test article will be formulated in an appropriate vehicle to be used for dosing in non-clinical studies. According to section 58.113 (a) of the GLP regulations[i]:

For each test or control article that is mixed with a carrier, tests by appropriate analytical methods shall be conducted

- *To determine the uniformity of the mixture, and to determine, periodically, the concentration of the test or control article in the mixture*
- *To determine the stability of the test and control articles in the mixture as required by the conditions of the study either (i) before study initiation, or (ii) concomitantly according to written standard operating procedures, which provide for periodic analysis of the test and control articles in the mixture*

To meet GLP requirements, suitable analytical methods must be developed and validated to determine the concentration, homogeneity, and stability of the test article in the vehicle. As for any analytical method development, the intended use of the method must be considered. For non-clinical dose formulation analysis, this would include the following considerations:

- The protein analyte and its physicochemical characteristics
- The vehicle for the dose formulation
- The protein dose concentration ranges to be measured
- The appropriate analytical methods that can address all of the above

Additionally, the definition of "stability" for non-clinical dose formulations is different from that usually adopted for pharmaceutical sciences-type applications. For a protein biologics dose formulation, "stability" refers to the preservation of total protein content after storage at a specified stability condition. Generally it does not refer to the measurement of chemical stability[iii] or biological activity. Therefore, the analytical method to be developed must be suitable and adequate for total protein concentration measurement, but it does not have to address the measurement of chemical stability or biological activity. If the chemical stability and biological activity of the protein in the dose formulation needs to be examined, it is suggested that these be evaluated separately from the method development related to total protein concentration determination in dose formulations.

The following case studies illustrate the approaches to selecting and developing analytical methods for total protein concentration determination in non-clinical dose formulations.

Case Study 1: A Monoclonal Antibody

The protein is a monoclonal antibody. The vehicle is a phosphate buffer, containing sodium chloride, sucrose, and Tween 80. The dose concentrations for the non-clinical study range from 1–10 mg/mL. A UV method is available for concentration measurement, using an extinction coefficient of 1.41 mL/(mg*cm) at 280 nm. For an absorbance range of 0.141 to 1.000, a linearity range from 0.100 to 0.709 mg/mL can be calculated from the extinction coefficient. For dose concentrations of 1–10 mg/mL, analysis can, therefore, be performed by diluting the dose formulations to within the linearity range for measurement. For this particular example, a simple UV method is a suitable method for measuring concentrations from 1–10 mg/mL of the protein analyte in the vehicle.

Case Study 2: A Monoclonal Antibody

The protein is a monoclonal antibody. The vehicle is a Histidine buffer, containing NaCl. The dose concentration to be used in the non-clinical study is 0.01 mg/mL. Similar to Case Study 1, a UV method is available and the extinction coefficient is 1.41 mL/(mg*cm) at 280 nm. However, the dose concentration of 0.01 mg/mL is outside the calculated linearity range of 0.100 to 0.709 mg/mL for the UV method. Therefore, a UV method would not be a suitable method for total protein concentration

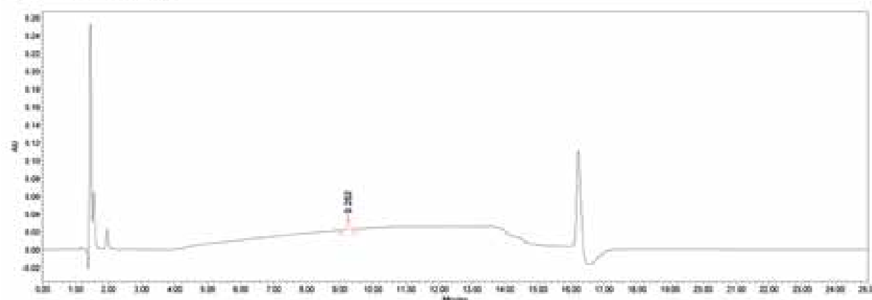
measurement. Instead, a size-exclusion HPLC method (SEC-HPLC) would be more appropriate. Detection can be conducted for the protein at a wavelength of 215 nm due primarily to contribution from the peptide bonds. Separation by size-exclusion separates the protein analyte from the components present in the buffer that could potentially interfere at 215 nm. As the absorption at 215 nm is greater than at 280 nm for a protein, a lower quantitation limit can be achieved. Based on method development experience in the author's laboratory, an SEC-HPLC method with detection at 215 nm for a monoclonal antibody in a similar vehicle can have a linearity range of 0.0025 to 0.02 mg/mL. For this case study, an SEC-HPLC method would be a suitable method for the measurement of dose concentration at 0.01 mg/mL.

Case Study 3: A Recombinant Protein

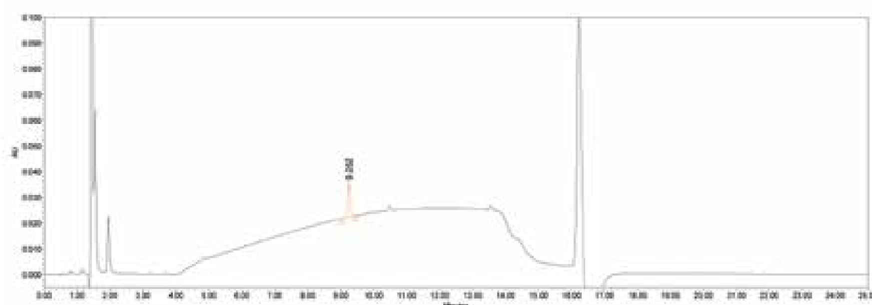
The test article is a non-glycosylated recombinant protein. The vehicle is saline, and the lowest dose concentration for the non-clinical study is 0.02 mg/mL. A UV method is available and the extinction coefficient is 1.4 mL/(mg*cm) at 280 nm. For an absorbance range of 0.140 to 1.000, a linearity range from 0.100 to 0.714 mg/mL can be calculated from the extinction coefficient. As the intended dose concentration of 0.02 mg/mL is outside the calculated linearity range of the UV method, it would not be a suitable method for total protein concentration measurement. Instead, a reversed-phase HPLC method (RP-HPLC) is a more suitable method. The absence of glycosylation in the protein allows for the main peak to be used for quantification, provided good resolution can be achieved. As described in Case Study 2, detection can be performed at 215 nm and a lower quantitation limit can be obtained. Based on method development experience in the author's laboratory, an RP-HPLC method with detection at 215 nm for a recombinant protein of similar size in the same vehicle can have a linearity range of 0.005 to 0.015 mg/mL. For this case study, an RP-HPLC method would be a suitable method for measuring the dose concentration at 0.02 mg/mL. A sample chromatogram illustrating the measurement of a similar recombinant protein diluted to a concentration of 0.01 mg/mL in a similar vehicle is shown in Figure 2.

Figure 2: Sample Chromatograms of a Recombinant Protein at a Dose Concentration of 0.02 mg/mL in Vehicle (diluted to 0.01 mg/mL) analysed by an RP-HPLC method. The recombinant protein peak is at 9.252 min.

(a) Full Chromatogram



(b) Expanded Chromatogram



Case Study 4: Mixture of Proteins

The test article is a mixture of proteins derived from a cell extract. The vehicle is saline. The dose concentrations for the non-clinical study range from 0.2 to 1 mg/mL, based on the total protein content in the cell extract. According to the certificate of analysis, the total protein content of the test article is determined by a Kjeldahl method[iv], which is capable of measuring the total protein content in a sample without the need for individual protein standards. Using the Kjeldahl concentration, calibration standards can be prepared and methods such as the Bradford, Bicinchoninic acid (BCA), and Lowry assays[ii] can be developed for total protein content measurement. Dose formulation analysis would be conducted by dilution of the sample

to within the linearity range of the method. In this case, an HPLC method would not be the most suitable approach, as dosing is performed by the total content of protein present in the mixture, and it is unnecessary to determine the concentration of each of the individual proteins.

Conclusion

When conducting non-clinical studies using a protein biologics test article, a good understanding of the protein's unique physicochemical characteristics, its characterisation as a test article, and the use of appropriate methodologies for dose formulation analysis, all contribute to ensuring the study meets GLP regulatory requirements, so that a well-executed non-clinical study can be included as part of a regulatory submission.

References

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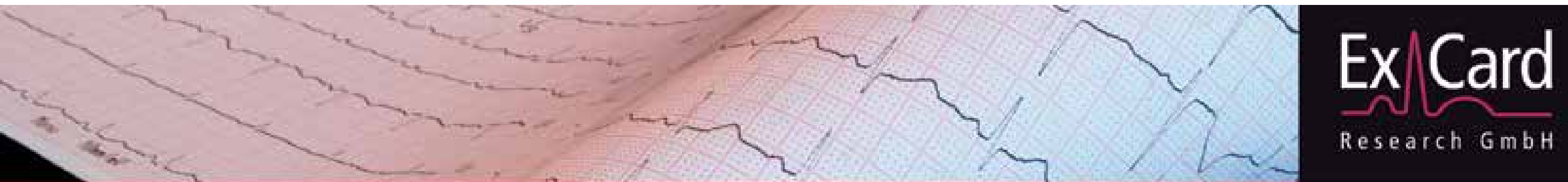

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