A Standard Protocol for Deriving and Assessment of Stability

Part 2 – Aseptic Preparations (Biopharmaceuticals) Incorporating Addendum on Antibody Drug Conjugates

5th Edition

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Document History	Issue date and reason for change
Edition 1	Issued October 2012
Edition 2	Issued December 2015 – update of techniques used for analysis if biopharmaceuticals, clarification regarding the use of published studies, statement on on-going stability assessment added, Appendix 1 for procurement assessment added and references updated
Edition 3	Issued April 2017 – update 5.5, one time point beyond proposed product shelf life, 7.5f addition of FTIR/Raman, 7.4 sub-visible particles counts allow use of volumes lower than BP Light Osbcuration and addition of reference, 11 on-going stability evaluation and introduction updated document in series
Edition 4	Issued August 2020 – 1. Addition of the Antibody Drug Conjugate addendum and references to it 2. Clarification regarding the use of biochemical binding assays for mAbs 3. Updated information of various analytical techniques including section to cover Imaged Capillary – Isoelectric focussing (icIEF) 4. Additional section of reports 5. Additional section on extrapolation to other container types
Edition 5	Issued June 2021 - 1. Updated following comments from MHRA on version 4 2. Review and update of references 3. Improved wording and clarity

1. Scope

Standards produced by the NHS Pharmaceutical Quality Assurance Committee and its sub-committees are produced with a distinctive yellow cover and are therefore known as Yellow Cover Documents (YCDs). This document is the second in a series looking at the stability of pharmaceuticals, and has been produced by the Pharmaceutical Research and Development Group.

Biopharmaceuticals incorporate a wide range of products such as vaccines, immunoglobulins, monoclonal antibodies, and cell and gene therapy products. This document intends to cover biopharmaceutical products that are aseptically manipulated and principally monoclonal antibodies and antibody drug conjugates, which are covered by the addendum, although many of the principles will also apply to other proteins and polypeptides. This document does not include reference to cell-based therapies (Advanced Therapy Medicinal Products), vaccines or blood components.

This document refers to the aseptic manipulation of licensed biopharmaceuticals and is not to be applied to products derived from first principles when all relevant ICH guidelines must be followed.

2. Introduction

Enabling preparation or procurement of ready to use biopharmaceuticals and in particular for monoclonal antibody (mAb), mAb fragment products or fusion proteins, is driven by both patient safety[1] and potential cost savings from more efficient product use.

Stability of the protein structure is an important feature in preserving the safety and efficacy of biopharmaceuticals. For example, any factor causing physical or chemical changes can alter the 3D structure and folding of the protein. Monoclonal antibodies are made up of four polypeptide chains (1300 amino acid residues) with two antigen binding sites that are critical to activity.

Due to the intricate nature of biopharmaceuticals the assessment of stability is highly complex and requires specialist input in order to interpret data from a multiple of orthogonal techniques, and to design robust stability trials.

It should also be borne in mind that stability trials on biologicals, and particularly stability trials on monoclonal antibody products, are expensive to run and therefore commission so there is likely to be a reluctance from suppliers regarding sharing of their raw data.

The Pharmaceutical Research and Development Group has taken a lead on the review of stability data for this product group and can advise the NHS where robust data exist to support product procurement, and also where a unit has sufficient data to assign an extended shelf life to its own preparations, including both preparation under Section 10 exemption and under the terms of a Specials Licence.

The principles informing this protocol are:

2.1 Information from manufacturers on aseptically prepared doses in their Summaries of Product Characteristics (SmPCs) is often limited, although the situation is improving. Generally, further stability data is not forthcoming from the relevant manufacturers although again there have been some recent softening in data sharing, particularly for biosimilars or where biosimilars exist.

2.2 Although published studies for monoclonal antibodies are improving in quality and robustness, often published data are of limited value to clinical practice due to a restricted range of analytical techniques, single or restricted ranges of concentrations tested, insufficient assessment of criteria, and insufficient detail of preparation methodology.

Studies published without appropriate peer review should not be used in the assignment of shelf life. However, studies published in robust peer reviewed journals can be used to form part of the stability picture although these studies must still be independently assessed against the standards in this document and against each local situation.

Manufacturing processes, final product containers and all consumables must be matched to the published study or SmPC, and further assessment of the in-house produced products including end of shelf life testing against a protocol covering the physical, chemical and biological characteristics of the product must be carried out. This, together with the published studies and assessment, should be assembled into a stability technical file for the product and subjected to expert review by someone with extensive knowledge in the field

2.3 Stability of biopharmaceuticals can be affected by different handling procedures as well as other factors such as choice of final container, amount of air present in the final container, and the amount of silicone oil in syringes. It is generally not appropriate to extrapolate data unless the criteria for handling of the product have been well defined and can be matched precisely.

2.4 Shelf life assessment requires a selection of orthogonal techniques including physical, chemical and biological assessment of activity[2,3,4]. Shelf life cannot be determined by one stability indicating assay for a single aspect but instead requires an evaluation of the available evidence for all necessary aspects of stability for the product.

2.5 Degradation routes for biopharmaceuticals are complex and can include chemical changes, conformational changes, aggregation, fragmentation and interactions with containers and excipients.

2.6 The expectation of the MHRA Specials Q&A[5] is that the product expiry should be based on scientific rationale, including test data. The document states that special attention should be given to shelf lives assigned and the methodology used for biologically derived products such as mAbs. The assigned shelf life must include a margin of safety from the stability data available. There should

also be a periodic review of the shelf lives assigned (see section 13). The manufacturer should be able to provide information to demonstrate its compliance with these requirements.

2.7 The principles that inform this protocol are applicable to monoclonal antibodies and, to an extent, other proteins and peptides.

2.8 All biopharmaceuticals should always be named using both their approved name and their brand name. It is not acceptable to extrapolate data to biosimilars or to other brands to those actually studied.

2.9 Antibody Drug Conjugates (ADCs) are conjugates of antibodies and drugs or toxins linked through a linker molecule and are, therefore, even more complex than other biological products. The activity of the antibody and of the drug alone does not prove clinical efficacy since the integrity of the bonding between the two components is critical for safe clinical use.

This group of products is dealt with in an addendum to this document, although the principles within the main body of this paper may be relevant when assessing the antibody component of the conjugate. Due to the complexity of these products and the additional risks associated with degradation, only studies which are fully compliant with this document will be accepted.

Procurement of aseptically compounded biopharmaceuticals should only be considered where either the shelf life assigned is within the SmPC or starting material manufacturers additional stability data (which has been subject to expert interpretation against this document on behalf of the NHS), or where the stability study and any additional data (e.g. end of shelf life testing) has been assessed as suitable in line with this document by experts in the field. Please see Appendix 1 for further guidance. The R&D Group Assessment Template for Biopharmaceuticals should be completed for all assessments of stability for this product group[6].

3. Biopharmaceuticals

There is no broadly recognised definition of biopharmaceuticals, biologics or biological medicinal products. In general, they are therapeutic proteins or polypeptides produced through biotechnology methods using a living organism. They are composed of a biologically active substance that is produced or extracted from a biological source and for which a combination of physico-chemical testing, and the manufacturing process and its controls, are required in determining its characteristics and its quality. The complexity of therapeutic proteins and their manufacturing processes makes the production of an exact copy impossible and hence there is always a degree of heterogeneity.

Biopharmaceuticals are developed using one or more of the following biotechnology techniques: recombinant DNA; controlled gene transfer / expression; and/or monoclonal antibody production. Once a biopharmaceutical is produced, a critical part of the manufacturing process is purification from cell culture. Typically, the product will undergo a series of purification steps, after which formulation and

sterilisation steps are performed in order to obtain the required active pharmaceutical product.

Biosimilars are a sub-set of biopharmaceuticals manufactured to have equivalent biological activity to an Authorised Reference Product. As stated above, the complexity of therapeutic proteins and their manufacturing processes makes the production of an exact copy impossible; therefore, there are no true generic forms. Even small and seemingly insignificant manufacturing changes could theoretically contribute to differences in protein folding, aggregates, and glycosylation, which might manifest clinically as altered efficacy, altered pharmacokinetics, or increased immunogenicity. Thus, heterogeneity between the same proteins from different manufacturers and even between batches from the same manufacturer cannot be avoided. The degree of heterogeneity does vary based on the structure. For example, Cetuximab is very heterogenic[7] whereas Natalizumab is much less so. The term 'biogenerics' has been used for these products but it is generally felt that 'biosimilar' is a more appropriate description and hence is more widely used. They are not identical to the comparator product therefore data cannot be extrapolated from originator products either to or between biosimilars.

The term 'Biobetters' is also used and refers to a biopharmaceutical with improved activity or reduced side effect profile compared to the reference molecule, and it is largely used as a marketing term. Biobetters should be treated in the same manner as other novel biopharmaceuticals necessitating the need for specific stability studies.

Variability is inherent in biopharmaceuticals due to their manufacturing process; this means that stability assessment, and particularly chemical stability, can be difficult.

Biopharmaceuticals in general and monoclonal antibodies specifically are used to treat a wide range of conditions. The condition being treated and the ability to monitor biological activity *in vivo*, for example using biomarkers, may also need to be taken into consideration when assessing the appropriateness of extended shelf life data.

In vivo data, patient response to treatment and adverse drug reactions should be monitored and reported in line with pharmacovigilance legislation[8].

The assessment of extended data for biopharmaceuticals is always a risk-based process and it should be noted that the longer the shelf life assigned the higher the risks of an undetectable but clinically significant change occurring in the molecule. For this reason, studies with a short but usable shelf life assignment may often result in a more robust assessment than those which seek to give excessive shelf life to the product.

4. Degradation pathways

Degradation of biopharmaceuticals can follow a variety of pathways including denaturation, fragmentation, aggregation, unfolding or misfolding, and chemical degradation to the amino acid residues. Adsorption onto surfaces or interactions with excipients can also affect the stability of the product.

Degradation can be influenced by a variety of factors including elevated temperature, exposure to light (especially UV light), inappropriate pH, removal or dilution of excipients, exposure to oxygen, shear stresses, excessive agitation and interactions with surfaces and interfaces. Considerations of shear stresses and agitation are also important when considering transportation between sites.

Increased temperature enhances the rate of chemical degradation processes and may also denature molecules which can lead to aggregation. Freezing can also lead to the denaturing of protein molecules.

Light and oxygen may cause oxidation of methionine, cysteine, lysine, histidine or tryptophan residues, may disrupt disulfide bonds, and may lead to unfolding or misfolding. All of these could result in a conformational change resulting in reduced activity or increased adverse effects.

Inappropriate pH will have a major effect on chemical degradation including:

a) Oxidation of methionine and tryptophan
b) Deamination of asparagine
c) Isomerisation of aspartic acid
d) Formation of pyroglutamic acid from glutamine

Protein molecules may associate to form low order oligomers, whilst denatured proteins tend to form aggregates leading to particulate formation. Aggregation can result from a large number of chemical or physical changes or from dilution of protective excipients. Some aggregate formation may be reversible and some will not be. Some studies have shown a decrease in particulate levels in the period between preparation and the SmPC assigned shelf life[9].

Fragmentation can result from re-arrangement of disulfide bonds causing disassociation. Formulated products are normally free from actual proteolytic activity.

Contact with metals, silicone oil and other excipients can also enhance degradation, which needs to be borne in mind when considering choice of storage containers.

The clinical impact of degradation may be seen as a decrease in efficacy and/or by increases in toxicity and immunogenicity.

There is a need to assess all types and routes of degradation as part of robust stability trials.

5. Stability Assessment

The stability of biopharmaceuticals is complex and needs to be assessed using a series of orthogonal techniques. These techniques must include stability indicating analytical methods to assure chemical and physical stability as well as confirmation of biological activity using a suitable biological or biochemical assay. Monographs for biopharmaceuticals are starting to appear in the European Pharmacopoeia (and are published in the British Pharmacopoeia) and this should be the first point of reference when designing a stability testing protocol.

When there are multiple mechanisms of action that contribute to clinical efficacy it may be necessary to apply more than one biological cellular assay or at least to ensure that the biological assay chosen is representative of the known mechanisms of action for a specific drug. It is acknowledged that for some mAb products the mechanism of action is not well understood and, in this case, the biological assay selected must undergo even more stringent scrutiny when assessing its applicability to the clinical situation.

For some biologicals, reference standards are publicly available from various pharmacopoeias for use in physico-chemical methods and/or biological potency. WHO International standards (IS) for potency are available from National Institute for Biological Standards and Control (NIBSC), part of the MHRA, including IS for etanercept, infliximab, rituximab, adalimumab and bevacizumab. Where it is not possible to obtain reference standards for biopharmaceuticals either the un-manipulated or freshly manipulated licensed medicine is often used as a reference. It is important that this reference has been stored in accordance with SmPC requirements and remains within its expiry date throughout the study, or that it is a secondary standard which has been compared to a primary standard which does meet these criteria.

The various techniques used in biopharmaceutical analysis are discussed further in section 6 below. Once the scope of a study has been defined then the process must be followed. All analytical methods require validation in line with ICH Q2[10], and if techniques selected are found to be unsuitable then the study should be abandoned and reconsidered. Any stability protocol must provide a clear technical/scientific rationale for the acceptance criteria, the sampling plan adopted, the assays used and the assays excluded. The context of other published information used to guide that rationale, such as European or UK Public Assessment Reports, should be critically evaluated in how they are related to the stability of the product under consideration before being used as a supporting rationale.

5.1. Diluents

The default diluent should be 0.9% w/v sodium chloride or 5% w/v glucose as specified in the product SmPC. Other diluents maybe added or supplemented if applicable and the rationale is set out.

5.2. Containers

Due to the complex interactions which can occur with biopharmaceuticals it is generally not acceptable to extrapolate data from one container type / manufacturer to another, and certainly unacceptable when these containers have different characteristics such as different materials or head space etc. Studies should be carried out in the specific containers and closure systems to be used in clinical practice. The method of container filling, including the type of syringes used for in-process handling, should also be clearly defined and should reflect normal practice as even this part of the process can have an impact on stability.

Ideally a stability study needs to be carried out in the actual volume of container to be used for routine supply, or if applicable in a range of relevant container sizes. However, this could prove prohibitively expensive and hence with expert assessment and understanding of the molecule and its potential instability, together with consistent handling (same proportion of air in the container etc.), it may be possible to extrapolate data to different container sizes. In the case of protein-based pharmaceuticals, because the interfaces between the solution and the plastic container, as well as those between the protein solution and the air headspace in the container, are places where unfolding and aggregation are more likely to occur, the higher surface area to volume ratio would be the worst-case scenario. The smaller the container size, the higher the surface area to volume ratio, and therefore a smaller container would be considered the worst-case scenario. Although container size may be varied the container used in the stability trial must be of the same composition as that used in practice. This hypothesis cannot be extrapolated to preparations stored in syringes where the actual syringe sizes used are required to be tested.

Container integrity should form an integral part of the stability assessment for all container types, and there is specific guidance for syringes used as storage devices below.

5.2.1 Syringes

Syringes used as storage containers must be fully validated including for microbiological integrity and physical robustness. Please refer to the 'Protocol for the integrity testing of syringes'[11] for further information.

The syringe and closure system should be fully defined and the data generated will be specific to this system. It is desirable to use luer lock syringes but two-piece polypropylene syringes may be required for some products even though past history indicates that there may be more issues with the integrity testing[12].

It is preferable that syringes, with the plunger attached, should not be filled to higher than 85% of their marked capacity, to prevent undue plunger movement and thereby potentially compromised microbial integrity during shipping and distribution: this is as outlined in 'Microbiological protocol for the integrity testing of syringes'[11] and national cytotoxic standardised specifications[13].

5.2.2 Infusion Containers

It is recommended that non-PVC containers (polyolefin) are used as first choice container. Data cannot be extrapolated to other container types and care should be taken when extrapolating to different manufacturers' containers because there is some evidence[14] that not all polyolefin bags behave in the same manner with all biologicals (see further advice in section 12).

Particular issues would be expected in extrapolating data to rigid or semi-rigid containers where there is more air present as this can increase oxidation but also the level of product agitation, and hence potential damage during handling and transport.

It is not currently common practice to administer biopharmaceuticals in other container types such as elastomeric infusers. However, if these are to be used then the study must be carried out in the specific container to be used clinically.

5.3. Storage Conditions

Due to the protein nature of the molecules concerned accelerated conditions are generally not appropriate for stability trials. Hence all stability trials must be 'real time' and 'real condition' studies.

Generally, only two storage conditions are required:

a) Refrigerated in the absence of light ($5^oC\pm 3^oC$)
b) Room temperature ($25^oC \pm 2^oC$)

For a refrigerator stored product, the in-use room temperature data can be obtained by storing for a period at room temperature at the end of the study, which would normally be limited to either 24 hours or 48 hours. This will help to reduce the costs of a study compared to an independent room temperature study being carried out.

If the product is likely to be exposed to light (e.g. during infusion) then the effect of exposure to continuous fluorescent light at room temperature should also be assessed. Further guidance will need to be sought on a case by case basis to assure that this assessment is undertaken appropriately[2].

This group of products is generally given by bolus or intermittent injection and hence it is not necessary to store the products close to the skin at body temperature (as it is for some continuous infusion products) and it is also not recommended that products are frozen as this could cause denaturing.

Products in ready to use form are in aqueous solution and hence apart from an assessment of the moisture loss from the container there is no need for control of humidity in the stability study.

5.4. Concentrations

Ideally each drug should be studied at clinically significant low and high concentrations. In this way if the drug shows a consistent stability profile it should be possible to interpolate to concentrations between the two studied concentrations. Note that with biological molecules there is some evidence of a tendency for lower concentrations to be less stable, which could be related to dilution of stabilising excipients or adsorption onto containers and components. It will not be acceptable to extrapolate beyond the range of concentrations studied and shown to be stable. Hence, where there may be stability issues at high or low concentrations it may be worth including a third intermittent concentration in case one of the highest or lowest concentrations shows instability during the study.

5.5. Storage Period / Study Length

Due to inherent variability in these molecules, for reconstituted products it is always best to manufacture or procure products with a shelf life which is applicable to the data but is as short as practicable. A longer shelf life does not necessarily mean a better product.

Because degradation can be complex and the implications of the degradants may not be well understood it is not possible to define a safe level of degradation to define the end of a study. It is typical to assign a short but practical study period in order to generate a shelf life of between 48 hours and 35 days, and shelf lives beyond three months should not be considered.

Note that it is not possible to extrapolate a shelf life beyond the study period. In fact, it is expected that the study should be extended to one time point beyond the proposed shelf life of the product, and that the shelf life assigned ideally should not exceed 80% of this final time point showing adequate stability. This approach is in line with that recommended by the MHRA Guidance for Specials Licence holders[5].

5.6. Sampling Strategy

It is recommended that each study includes at least four sampling points in addition to the baseline (T=0) data to be spread over the study period.

Due to the complexity of the analytical methods a different strategy can be employed to that for standard stability studies, whereby, for suitable prospective stability studies, samples are prepared at intervals (using the same batch of raw material) and stored appropriately in advance of the test date, the testing of all samples then takes place on the same day. Note that freshly prepared samples for T=0 must be included and an unmanipulated sample and if possible a reference standard or additional batch of product for use as a reference preparation should be included.

This approach is suitable for studies requiring stability data for licensed products transferred from the licensed container to a different storage device; however, for products which require further dilution or manipulation, additional precautions to limit the variability between test samples may need to be employed, such as weighing the containers before and after addition of the drug.

5.7. Sample Numbers

Due to the complexity and inherent variability of this group of products it is strongly recommended that the stability studies include **three independent batches at each of the concentrations studied**. As a minimum, at each concentration each independent ‘batch’ must be a separate container (i.e. three independent containers for each concentration). The ideal is for three truly independent batches of starting material to be included in the study, as expected by ICH. This will allow assessment of stability variability related to product heterogeneity and any variations in the manufacturing process.

For relevant techniques, such as chromatography, each of these batches should have a minimum of three replicates tested at each time point. A risk-based approach is acceptable, based on confidence in the data and operational factors. Bracketing and matrixing may be used in order to carry out a cost-effective but meaningful stability study. For some assay types (see below) more replicates may be necessary.

5.8 Preparation / production process

The preparation / production process for biopharmaceuticals and their transport are all critical factors for their stability; the stability trial samples must have been handled in the same way as the product will routinely be handled during production. Any changes to the production process must undergo a robust change control process with a full impact assessment. There needs to be a very good understanding of the molecule and its stability in order to make such assessments.

Changes in syringes used in the process and needles or vial access devices can be critical in terms of compatibilities with individual components, and also consideration of shear pressures that the molecule is subjected to.

6. Testing Protocols

The minimum testing protocol should include:

a) Colour, clarity and particulates
b) pH
c) Chemical stability
d) Physical stability
e) Assessment of sub-visible particle levels for example light obscuration particle counting, Microflow Imaging or other particle size analysis
f) Biological activity (cell based or biochemical assays as applicable)
g) Assessment of degradation and aggregation together with the clinical impact of degradation / aggregation products.
h) Representative assays for critical excipients including tissue permeability enhancers for sub-cutaneous presentations

Additional parameters which may need to be considered include:

a) Moisture loss (particularly for infusion bags – this can be carried out by weight check after storing at ICH low humidity storage conditions)
b) Container leachables
c) Additional excipient concentrations

Note that each of these assessments may include a series of orthogonal techniques and not a single analytical technique.

7. Test Methodology

There needs to be good understanding of the inter-relationship between the tests selected and the interpretation of the data generated. The following techniques may

be of use in assessing the stability of biopharmaceuticals in 'ready to use' presentations.

7.1 Forced degradation

In order to demonstrate the stability indicating nature of all assay types used forced degradation studies should be conducted. Bearing in mind the nature of the molecules these studies need to be carefully designed as dramatic changes in temperature, pH, oxygen level etc. could have an unrepresentative effect on the molecular structure of proteins.

The following methods can be considered:

a) Controlled change in pH
b) Realistic elevated temperature – high temperatures are likely to cause total denaturing of the structure
c) Exposure to several freeze thaw cycles
d) Exposure to UV light
e) Agitation

The fitness for purpose of the validated method used in the stability protocol is to be demonstrated. This can be achieved through forced degradation studies; the techniques used must be justified.

Note that storage at controlled room temperature, i.e. 25°C±2°C, for an extended period may result in sufficient degradation to demonstrate the stability indicating nature of an assay. Samples taken throughout an extended period can lead to preparation of stability profiles for the molecules and that can provide good evidence the techniques used will detect important changes, and will also help put any changes seen within the actual study into perspective. This may not, however, be a realistic method for the initial assay fitness for purpose assessment.

7.2 Visual Characteristics

Appearance of solution, colour, clarity and absence of visible particulates.

7.3 pH

pH is crucial to stability of biopharmaceuticals and hence it is indicative of unfavourable stability conditions. Changes in pH during a study are also indicative of a lack of stability.

7.4 Particulates

Protein aggregation will eventually result in particles large enough (ca. 10µm) to be detected by standard sub-visible particle testing equipment such as a light obscuration liquid particle counter, however if carried out to the Pharmacopoeial standard tests this technique does need relatively large sample volumes. There is evidence that a smaller sample volume will provide equivalent accuracy in terms of particle level analysis[15] and therefore smaller sample sizes for particle analysis may

be acceptable. The USP<787> Subvisible Particulate Matter in Therapeutic Protein Injections[16] allows the use of smaller test product volumes and smaller test aliquots to determine particulate matter content.

Microflow Imaging may be a better option for biopharmaceuticals since this technique is more sensitive (1µm) and can differentiate sources of particles, for instance separately identifying aggregates and silicone oil droplets which could be vital when looking at products in syringes. Another advantage of this technique is the small sample volume required.

In some instances, particularly with the small sample volumes available, simpler techniques such as fluorescent magnification viewing may need to be employed. Techniques including Nanoparticle Tracking Analysis and Resonant Mass Measurement have also been used for the study of biopharmaceuticals.

Unless separate containers are being used for sub-visible particle analysis at each time point, the introduction of particles caused by the sampling process must be considered.

Additionally, sub-visible particle levels may be assessed using some of the physico-chemical testing (size exclusion chromatography, dynamic light scattering) outlined below.

7.5 Physico-Chemical Stability

Analytical techniques are generally required which can detect aggregation below the limit of detection of the optical methods described above and which will also detect other physico-chemical changes such as conformational stability.

A variety of techniques are used and normally a combination of these will be required to give robust information on the physico-chemical stability of a formulation. However, physical methods alone are not good at detection of neutral or low molecular weight changes.

Further information including detailed analytical methodology is to be found in the USP chapters covering the analysis of biopharmaceuticals[17,18,19].

a) Size Exclusion Chromatography

This is a chromatography technique where molecules are separated by size because different sized molecules pass through a column at different rates allowing for separation. There needs to be about a 10% change in molecular weight in order to detect a separate peak. This technique is good for separation of dimers and aggregates.

By using this technique an assessment can be made of higher protein structure, adsorption, physical changes, size distribution, aggregates and oligomers. It can also provide for quantification / assay of the active molecule and a range of degradants, although any degradants cannot be characterised using this technique.

b) Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) measures the hydrodynamic radius as the equivalent size of a theoretical sphere and is another method for detecting high molecular weight aggregates; however, it cannot detect small changes such as dimerization as there needs to be at least a doubling of particle size for differentiation. DLS can, however, detect relatively small quantities of higher-level aggregates.

c) Weak Cation-Exchange Chromatography (CIEX) or Capillary Zone Electrophoresis (CZE)

Proteins are charged molecules due to the ionisable side chains and can, therefore, be separated by differences in their charge. Charged variants of protein products can be monitored using ion-exchange chromatography (IEC) techniques such as CIEX. Initially proteins bind to the exchange matrix displacing cations (normally Na+). Either a pH or ionic strength gradient is applied and separation takes place based on the protein charge. Degradation of the side chains and other structural changes will alter the protein charge and hence will influence retention time. CZE offers a reliable alternative to IEC for the analysis of charge heterogeneity by separating according to the analyte's net charge and hydrodynamic radius, introducing an additional size-based element to the separation.

d) Capillary or Flat Bed Gel Electrophoresis

Both capillary and flat bed gel electrophoresis are methods of gel electrophoresis where a high voltage charge is applied and protein molecules are separated based on their size to charge ratio. Both methods also enable the analysis of proteins under reducing and non-reducing conditions to determine the purity, degradation and molecular weight of the protein. Degradation of the protein will result in the detection of separate degradant bands or peaks.

Polyacrylamide Gel Electrophoresis (PAGE) is most commonly used in the separation and analysis of protein molecules. Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS – PAGE) detects degradation by band smearing and is often more qualitative than quantitative. The sensitivity of SDS-PAGE is dependent upon the protein stain that is used, with silver staining techniques being far more sensitive than Coomassie blue staining techniques.

Capillary Electrophoresis-SDS (CE-SDS) has emerged as a replacement to SDS-PAGE to provide a quantitative approach for the determination of the size heterogeneity and purity of biological products. CE-SDS separates SDS-labelled protein variants by a sieving matrix in a constant electric field, with the advantage of providing enhanced resolution of closely related size-variants and accurate quantification of proteins and their degradants, also the detection of non-glycosylated forms of monoclonal antibody proteins.

e) Imaged Capillary – Isoelectric focussing (icIEF)

Imaged Capillary – Isoelectric focussing (icIEF) is an alternative method to CIEX and is a highly resolving technique for separating different molecules by differences in

their isoelectric point. Analyzing charge variants of therapeutic proteins is critical for characterizing and monitoring quality attributes of antibodies. Charge variants include deamidation, formation of N-terminal pyroglutamate, aggregation, isomerization, sialylated glycans, antibody fragmentation, and glycation at the lysine residues. It considers surface-exposed and internal amino acids, with no loss of resolution due to hydrophobic interactions[20].

f) Circular Dichroism

Circular Dichroism (CD) spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light that arise due to structural asymmetry. The absence of regular structure results in zero CD intensity, while an ordered structure results in a spectrum which can contain both positive and negative signals.

Biological molecules exhibit CD due to their dextrorotary and levorotary components. More importantly the protein secondary structure will impart a distinct CD to its respective molecules. Therefore, proteins have CD spectral signatures representative of their structures.

Secondary structure can be determined by CD spectroscopy in the "far-UV" spectral region (190-250 nm). The chromophore is the peptide bond, and the signal arises when it is located in a regular, folded environment.

Certain aspects of tertiary structure can be detected in the "near-UV" spectral region (250-350 nm). At these wavelengths the chromophores are the aromatic amino acids and disulfide bonds, and the CD signals they produce are sensitive to the overall tertiary structure of the protein.

g) Infra-Red FTIR and Raman spectroscopy

FTIR and Raman spectroscopy are also often used for structural elucidation of biopharmaceuticals. Vibrational spectroscopy is an ideal technique for study of higher order protein and FTIR is sensitive to secondary and tertiary structures and sometimes higher structures. A validated database of protein structures is required in order to use the technique, and there are various spectral characteristics which must be met in order to validate an individual spectrum.

FTIR can be a complementary technique to CD although it does give better cover to the β sheet structure and hence has advantages for analysis of monoclonal antibodies where this predominates.

7.6 Chemical stability

a) HPLC

Traditional HPLC (HPLC-UV or uHPLC-UV) may have a role with some biopharmaceuticals. Often selective fragmentation (digestion) has to take place ahead of HPLC analysis, and detection may use a variety of techniques including

UV, mass spectrometry, N-terminal sequencing and amino acid compositional analysis.

b) Ultraviolet Spectrophotometry

The extinction co-efficient for the product can be determined at a specific wavelength (often around 280nm) and this is used to provide an indication of protein content although the technique is non-specific. It can be used to assess adsorption of protein molecules onto surfaces but it may not differentiate intact from partially degraded protein.

c) Mass Spectrometry (LC-MS)

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles for determining their elemental constitution, and for elucidating the chemical structures of molecules. MS works by ionizing chemical compounds to generate charged molecules or molecular fragments to facilitate the measurement of their mass-to-charge ratio. MS can be used as an assay for the active protein and for the detection and characterisation of degradants. MS detectors can be linked to HPLC and uHPLC liquid chromatography separation systems.

There are three types of mass spectrometry of use in the analysis of biopharmaceuticals.

First, whole mass analysis (LC-MS), which can be run on the native protein (non-reduced) or on a reduced form, and which can detect glycosylation, oxidation, deamination and fragmentation. It is also useful in the identification of fragments and determination of likely toxicity and immunogenicity properties.

The second technique is differential peptide mapping which uses a proteolysis stage ahead of the LC-MS analysis. This technique can measure the rate of chemical degradation for specific peptides for which limits can be set. Protein digestion is a critical and multi-step stage in this analysis which can be complex and the use of automated platforms may be advantageous in order to obtain a more consistent digest. This technique can identify levels and sites of many chemical reactions including: glycosylation; point mutations; disulphide bonds; deamination; oxidation; and levels of impurities.

Thirdly, there is the technique of amino acid sequencing that involves proteolysis followed by LC-MS/MS, and this technique can identify specific sites of chemical degradation and can be used to evaluate potential toxicity and immunogenicity.

Techniques based on mass spectrometry detection are developing rapidly for use in the field of biopharmaceutical analysis including linkage with Capillary Electrophoresis to provide separation and detection, for example CESI-MS (Capillary Electrophoresis – Electrospray Ionisation – Mass Spectrometry).

7.7 Biological activity

Assessment of biological properties constitutes an essential step in establishing an understanding of the stability profile under specific conditions. The technique should be relevant to the specific biological activity that enables the product to achieve its defined biological effect. Where there are multiple mechanisms of action contributing to clinical efficacy it may be necessary to apply more than one biological assay[22].

Results of biological assays should be expressed as relative to the standard preparation being used as the reference.

There are three main types of biological assays:

a) Biochemical assays

These measure biological activities such as enzymatic reaction rates or biological responses induced by immunological interactions.

Enzyme Linked Immuno-Sorbent Assay (ELISA) is a method that assesses the antibody-antigen binding of the molecule but is unlikely to detect structural changes which do not impact on the binding sites such as changes to glycosylation. The technique does not indicate or represent integrity of the biological response, binding does not demonstrate functional activity, and the two may not necessarily correlate. Degraded molecules may be immunoreactive and bind to their target antigen but might not elicit a functional response and hence a cell-based assay is always preferable.

Alternatives such as Mesoscale Discovery (based on a combination of electro-chemiluminescence detection and patterned arrays), Biacore (surface plasmon resonance (SPR)), and Gyrolab (offering a broad spectrum of immunoassay formats) may also be used.

Binding assays should be seen as complimentary to full cell-based assays. However, where the total action of the protein is in binding to / blocking specific receptors then an antibody-antigen binding assay may be suitable to demonstrate activity without the need for a full cell-based assay.

For example, Nivolumab and Pembrolizumab are monoclonal anti-programmed cell death-1 (PD-1) antibodies which block its interaction with ligands PD-L1 and PD-L2[23,24]. Even in these cases a cell based assay may add additional information to the study and hence is desirable.

It should be noted that for binding assays a trend in either direction (increased or reduced binding) should be treated as a sign of molecular instability and decisions made accordingly.

b) Cell culture-based assays

These assays measure biochemical or physiological response at the cellular level. A cell-based assay needs to show that the molecule will have the defined biological

activity for its function, hence each assay is likely to be specific for one particular aspect of biological activity and two or more cell-based assays may be necessary to assess products with complex mechanisms of action, for example a monoclonal antibody may engage two effector functions e.g. Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) and Complement Dependent Cytotoxicity (CDC).

For some biopharmaceuticals the details of the biological activity studies used in their licensing have been published, in which case the published methodology should be followed for the biological arm of the study.

In cases where there are no published methods, a suitable method must be designed and fully validated in line with ICH guidelines[4,11]. Understanding the biological activity at a cellular and molecular level is of paramount importance when designing such studies. A valid cellular process to measure the functional activity must be determined, for example expressing certain receptors. An appropriate cell line then needs to be chosen which must express the relevant receptors.

A well-executed assay that has been validated and is robust is expected and hence the cell-based assay selected should allow for an acceptable level of reproducibility.

Note that some factors which influence cell death may not be able to be simulated *in vitro.*

The cellular response to be measured also must be determined and may include cell signalling (e.g. levels of phosphorylated Akt), cell death (apoptosis – measured by flow cytometry) or cell proliferation (measure of metabolism e.g. MTT assay or preferably using soluble formazan dyes).

The ability to establish a good dose response curve is a vital part of the assay validation, and the BP chapter on statistical analysis of results of biological assays[25] should be consulted for further guidance in this area.

It is usual to use a high number of replicates for cell-based assays to allow for inherent variation in assay performance and to improve the robustness of the results obtained.

Other considerations include the stability of the actual cell line to ensure a consistent response across the study, and a good understanding of the methodology including validation to demonstrate that the cell-based assays will correlate with *in vivo* activity. Appropriate controls should be incorporated to validate the assay and should include positive reference standards in addition to negative controls.

c) Animal based assays

These assays measure the animal's biological response to the product. This type of assay is unlikely to be used in assessment of stability for an aseptically manipulated licensed product; animal models are not useful in assessing humanised antibodies due to the likeliness of immunogenic response. These are also strongly discouraged based on the 3R (Replacement, Reduction and Refinement) principles as replacement techniques are generally available.

7.8 Excipients

Stability of important excipients can usually be carried out using traditional stability indicating HPLC assays. Excipient instability may have a marked effect on the stability of the active moiety itself. Excipients critical to the successful clinical use of the product including tissue permeability enhancers (e.g. hyaluronidase) must also be included in the stability study and may need to take the form of an activity based (enzymatic) assay.

8. Acceptance Criteria

Acceptance criteria are a lot more difficult to define than for small molecules. It must be borne in mind that the coefficient of variation on biological assays is likely to be higher than that seen for traditional physico-chemical assays, and hence the biological assay data will require close scrutiny alongside the physico-chemical assay data and other factors.

Acceptance criteria should be supported by justifiable rationale on a case by case basis. A sufficient amount of data must be generated by the stability study in order that the stability profile of the product can be sufficiently understood. Subject to the capability of the specific assay technique, it would not be expected for the acceptance criteria for the physico-chemical testing to exceed 5% loss in active protein or any degradant peaks to exceed 2% relative to the main peak. All indicators of molecular instability should be of concern due to the likely lack of understanding as to molecular changes occurring and the clinical implications of this.

Aggregates, degradants and other impurities detected may be of known structure, partially characterised or unidentified. Where sufficient quantities are present these molecules should be characterised where possible and their impact on biological activity, immunogenicity or other undesirable affects should be assessed. Detection and evolution of new impurities at any stage of the stability study should be considered an indication of stability failure.

There should be no physical change in appearance and no significant change in pH (defined as a change of >0.5 pH unit). Biological or biochemical assays should broadly support the data generated by the other techniques. If other techniques indicate stability but there is a significant reduction of biological activity then this should raise concern. Failure to comply with any single acceptance criterion during a stability study should be regarded as a sample being out of compliance / specification and indicative of instability. The bioassay should be sufficiently robust that a significant reduction is an indication of loss of activity and a stability failure and not a reflection of the potential performance of the assay. Specific acceptance criteria have not been included in this document, however, acceptance criteria should always be scientifically sound and justified in the study protocol. Data should be assessed for significant drifts or trends throughout the duration of the study. For biopharmaceuticals for which the SmPC states a 24-hour shelf life for the diluted product, the initial test point of a stability study which is carried out on the day of manufacture (T=0) provides a suitable reference point as it falls within the SmPC assigned shelf-life for the diluted product.

It should be understood that when the manufacturer establishes acceptance criteria for product stability it is in the holistic body of total knowledge ranging from population pharmacokinetics to batch manufacturing and development history through an entire clinical and post marketing programme. The challenge of understanding the stability of a reconstituted product without any of this background body of knowledge should not be underestimated. All acceptance criteria must be scientifically justified at the start of the study and presented within the study protocol.

8.1 In-use storage

It is important to understand the use of the product as well as its storage in clinical areas. If light sensitivity has not been assessed during the stability study then the product should be provided in light protective packaging with the instruction to keep the product protected from light.

Similarly, in-use temperatures must not exceed those studied in the stability testing. It is important that robust change management is used if making changes to clinical protocols and particularly if the result is an increase in infusion time.

A period of 24 hours or 48 hours at room temperature at the end of the refrigerated study period should give an indication that the product is stable during infusion and also will add confidence to the data in the refrigerated study.

9. Statistical Concepts

Where appropriate statistical analysis should be applied to the quantitative data, however the shelf life decision is likely to be based on a review of all the data generated from the full range of techniques used. Statistical analysis must be carried out by someone with sufficient knowledge and understanding of biopharmaceutical drug stability and statistical concepts. British Pharmacopoeia 2021 SC IV G. Statistical Analysis of Results of Biological Assays and Tests (Ph. Eur. general text 5.3)[25] can be consulted for further advice.

10. Stability report

A detailed stability report should be produced at the end of the study, which should include the protocol, a discussion of all assay methods and justification for their use and their suitability and should clearly set out and justify the acceptance criteria assigned. All data should be presented including all replicates so that those data can be fully assessed.

The report should also justify any omissions in the protocol, the testing programme or data availability and explain any non-conformances such as out of specification or out of trend results.

All reports for use within the NHS should be reviewed and approved by a person with specific expertise and authorised to do so by the NHS Pharmaceutical Quality Assurance Committee. Approved data together with any limitations to specific suppliers or processes is maintained in ‘Summary of biopharmaceutical stability data

reviewed and available extended shelf-life data - Reference for NHS Procurement' produced by the NHS Pharmaceutical R&D Group[21].

11. Change Control

There must be robust change control processes in place and any changes to the containers, components and processes or in the manufacturing site or post manufacture handling must be subject to a full impact assessment on the stability assigned to the product. This assessment may include the need for end of shelf life assessments for physical, chemical and biological stability or a full repeat of the stability study.

12. Container extrapolation

Where sufficient evidence exists, it may be possible to extrapolate data to similar container types, for example a different manufacturer's polyolefin bag. In the case of biosimilars where different manufacturers have tested their product in different bags a case may be made to extrapolate data for a specific biosimilar to a similar polyolefin bag. If this is done, a further safety margin to the shelf life assigned is recommended.

Extrapolation from flexible bags to semi-rigid containers or to bags with needle free access devices is to be discouraged unless there is evidence that this will not impact on the product stability (for example where a spike needle free access device has been used in the study for removal of samples). Extrapolation to totally different container types (e.g. from polyolefin bags to syringes or elastomeric devices) is never acceptable.

13. On-going stability evaluation

Biopharmaceuticals have an inherent heterogeneity and can be considered as a mixture of closely related molecules. Authorised products are also subject to manufacturing variations and many marketed biopharmaceuticals have been subject to multiple regulatory variations in their life-span. These changes are assessed by the EMA and significant changes may require clinical trials[22]. For an aseptic unit these changes go largely under the radar, however they may impact on the validity of extended shelf life data. For this reason the extended shelf life data should be routinely reviewed and additional evidence such as annual end of shelf life testing can add significantly to the confidence that such data are still relevant.

Pharmacovigilance reporting for biopharmaceuticals is of paramount importance and if reporting an Adverse Drug Reaction involving a biopharmaceutical careful note should be made of the batch number, and also for aseptically compounded products the shelf life assigned and the period through its shelf life that the product was administered.

Glossary

Biopharmaceutical – A therapeutic protein or polypeptide produced through biotechnology methods using a living organism.

Biosimilar – A biopharmaceutical which has been manufactured to have equivalent biological activity to a branded biopharmaceutical.

Denaturation – Alteration or break down of the secondary and tertiary structures of a protein caused by disruption of bonding interactions which normally maintain this.

Fucosylation – A specific type of glycosylation whereby fucose sugar units are added to a molecule.

Glycosylation – The addition of a carbohydrate moiety to a molecule. Protein glycosylation is a critical step in the manufacture of monoclonal antibodies and in determination of their tertiary structure.

ICH – The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use.

Monoclonal antibody (mAb) – A monospecific antibody made by cloned immune cells, which will bind to a specific site.

MTT assay – Colourimetric assay for measuring the activity of enzymes based on reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan dye. Note the MTT is an insoluble formazan dye and this tends to increase assay variation, soluble formazan dyes are generally a better option.

Oligomer – molecule that consists of a few monomer units. Dimers, trimers, and tetramers are all oligomers. (c.f. a polymer which, in theory, consists of an unlimited number of monomers).

Phosphorylated Akt – Phosphorylated form of Akt which is a specific protein kinase that plays a key role in multiple cellular processes including glucose metabolism, apoptosis, cell proliferation, transcription and cell migration.

SmPC – Summary of Product Characteristics.

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22. ICH Q5E Comparability of Biotechnological / Biological products subject to Changes in their Manufacturing process www.ich.org/products/guidelines.html

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Appendix 1

Checklist for the assessment of stability data for procured aseptically prepared biopharmaceutical products (Specials)

The following checklist is provided as a quick guide to assessment of the suitability of procured aseptically compounded (from licensed starting materials) biopharmaceutical products from the stability assessment viewpoint. This should be used alongside other assessment tools for unlicensed products.

Preparation:…….

Supplier / Manufacturer:……

1) Formulation	1.1) Is the formulation specified in the product specification including any concentration restrictions	Yes (go to 1.2) / No (return to supplier for specification)
	1.2) Is the formulation fit for purpose and for the patient / patient group	Yes (go to 1.3) / No (source a suitable formulation)
	1.3) Is the preparation made in accordance with the SmPC	Yes / No (Record and proceed)
2) Shelf life assigned	2.1) What shelf life is assigned by the manufacturer	
	2.2) Is the shelf life based on the recommendations in the SmPC	Yes (go to 3) / No (go to 2.3)
	2.3) Is the shelf life based on a specific stability study (in-house or supplied by starting material manufacturer)	Yes (go to 3) / No (go to 2.4)
	2.4) Is the shelf life based on an expert (in the field of biopharmaceutical analysis) assessment of stability based on a published study or other information (extrapolation)	Yes (Go to 3)/ No (Ask supplier for more information or source another supply)
3) Expert interpretation	Ensure that the study is assessed by an independent expert in the field as being compliant with this document. Is a margin of safety applied?	A list of assessed studies is available[21]. Apply a suitable margin of safety if this has not been applied to the data

Summary of risks

Assessment of stability study for

……..

The data supplied: Provides assurance that the product will be suitable, safe and efficacious / Does not provide suitable assurance

Outcome approved:…….Date:……

Additional risk reduction measures

Addendum

Antibody Drug Conjugates

Background information

Monoclonal antibodies are now fully established in medicine across the developed world. They are used to treat a broad range of conditions and are generally safe to handle; there is guidance available as an NHS YCD, Guidance on the safe handling of Monoclonal Antibody (mAb) products published in 2015 [26].

Conjugating toxic compounds to antibodies was first proposed by Paul Ehrlich at the beginning of the 20th century. Ehrlich reasoned that if a compound could be made that selectively targeted a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity. The concept of a 'magic bullet' is now being realised through Antibody Drug Conjugates (ADCs), and there are currently four licensed ADCs in the UK and many in clinical trials and development.

It is important that pharmacy staff recognise ADCs where they are supplied as licensed products but also as IMPs or for compassionate use. These molecules are different from standard monoclonal antibodies and indeed from traditional cytotoxic drugs.

Structure and function

There are 3 distinct components of the ADC used in the oncology setting (ADCs are now being developed as a targeted approach for other medical conditions and these are not currently within the scope of this addendum):

- A **mAb** – at the core of the ADC is the highly selective monoclonal antibody (mAb), which is specific for an antigen that has restricted expression (tumour-associated) or no expression (tumour-specific) on normal (healthy) cells. The mAb is the key to internalisation of the ADC, facilitating the endocytosis of the ADC inside the target cell[27].

- A **potent cytotoxic agent** – known as the "warhead", generally a small molecule drug with a high systemic toxicity, designed to induce target cell death after being internalized at low intracellular concentrations[28,29]. Because of this, typical warheads are highly toxic, up to 4000 times more toxic than standard cytotoxic chemotherapy. The warhead is attached to the mAb via a linker and the warhead and linker are collectively termed the "payload".

- A **linker** stable in circulation, but not in cells – the linker has the function of binding the warhead to the mAb and keeping it bound until the ADC is internalised into the cell where cleavage takes place and the warhead or payload (warhead and linker)

is released. The linker is typically linked to the mAb via a cysteine, lysine or an engineered amino acid residue, although other forms of attachment are possible[30]. The linker does not introduce any activity to the ADC but is critical to efficacy and stability of the ADC in its formulation[30].

Note that some ADC's linkers (e.g. Kadcyla) are not cleaved but instead rely on proteolysis by lysosomal enzymes to release active amino acid-linker-warhead conjugates[31].

Stability Considerations

ADCs are heterogeneous in nature; this is due to variations in mAb structure (all mAbs are heterogeneous in nature and liable to alterations with changes to the manufacturing processes) as well as variation in linkage of the toxin. As with conventional mAbs stability of ADC's will be potentially affected by:

- Storage temperature[32]
- Changes in pH[32]
- Physical agitation[32]
- Light[32]
- Handling during compounding and shearing stresses during transfer[32]

In addition, the process of attaching a payload will lead to structural changes potentially changing physico-chemical stability, Fc and Fab binding, and shelf life. Studies have shown that an ADC can be susceptible to a higher rate of aggregation when compared to its parent mAb[31,33,34].

Pharmacy staff reviewing stability data for ready-to-administer doses of ADCs will be faced with a complex array of data from highly specific techniques. As for mAbs, a combination of orthogonal analytical methods is used. The standards outlined in the main body of this document regarding justification of techniques used, acceptance criteria and statistical analysis are also applicable to products covered by this addendum.

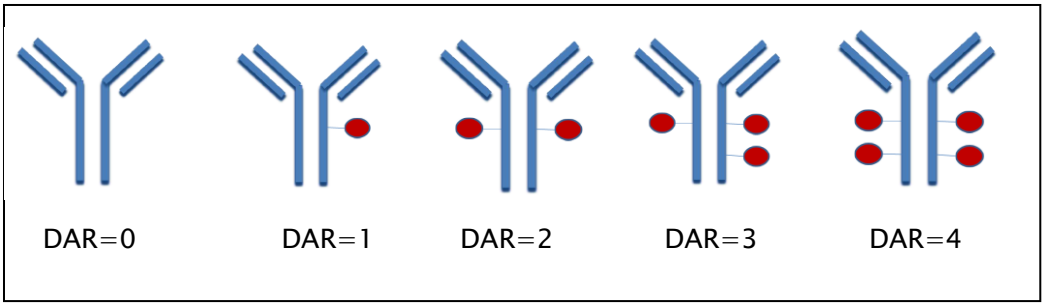
In addition to the minimum testing protocol required for the stability testing of mAbs (Section 6, main document), specific testing is required to assure quality parameters are met throughout the shelf life of the ADC in its formulation. Testing focuses on 3 key parameters:

(i) Drug Antibody Ratio (DAR)

A critical attribute of an ADC, the DAR indicates the average number of drugs that are conjugated. The number of DAR variants with an ADC will generally vary from 1-8[35]. A preparation will contain a mixture of DAR variants; overall DAR (DAR_{av}) is the mean DAR of the mixture. The DAR determines the amount of payload that can be

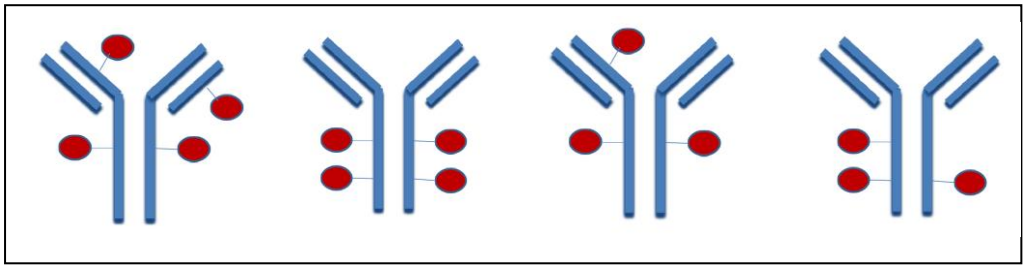
delivered to the cell and can directly affect both safety and efficacy[31,36], and the stability and pharmacokinetics[31,34].

DAR profile refers to the relative % each DAR variant constitutes the DAR_{AV}. For example, an ADC that was 50% DAR 2 and 50% DAR 6 would have a DAR_{AV} of 4, but its profile is 50:50 DAR 2:6.



(ii) Distribution of Payloads (DOP)

The payloads may be linked to the mAb in a variety of places dependant on the specific linkage mechanism, and payloads bound in the Fab and Fc regions can impact binding and hence activity[30]. The DAR profile can be further investigated by separating the mAb chains and/or fragments, and measuring payloads bound to specific regions on the mAb. However, once manufactured, new linkers cannot be attached to the antibody, thus the sterically hindering positional isomers that can be problematic at a manufacturing stage are not a concern for ongoing stability.



(iii) Unconjugated Drug (Free Drug/payload)

Traces of the unconjugated (free) drug/payload and also toxin linked solely to the linker are likely to be present in the pharmaceutical product from manufacture and may increase on manipulation and aging. The free drug may be a critical parameter for patient safety where it has high potency/extreme toxicity and limits will be defined within the MIA holder's product specification but are unlikely to be publicly available.

Free drug concentrations will be inversely related to the DAR and inherent stability of the linker chemistry, as the DAR decreases the free drug/drug related species will increase. Any increase in free drug and/or drug related species should be considered a serious problem unless robust data are available to confirm lack of systemic toxicity linked with identified degradants.

Interpretation of free drug level increases on storage can be difficult without understanding the specification limit for this criterion. The use of off-target cellular assays could help provide toxicity data to contextualise the changes in free drug concentration.

Test methods

The Testing Protocols and Test Methodology described for the analysis of mAbs in Sections 6 & 7 of the main document are also applicable for the analysis of ADCs, since the degradation processes monitored by these tests are relevant to both mAbs and ADCs, although it should be noted that even those methods that are routinely used for analysis of mAbs (e.g. SEC) may require modification for use in the analysis of ADCs.

The inherent complexity of the mAb component is further complicated in ADCs due to the added variability introduced by the number of drugs attached to the antibody resulting in heterogeneous populations; this can lead to altered physical and chemical characteristics of the ADC compared with the parent mAb, presenting additional stability implications and analytical challenges which must be addressed to ensure the stability and safety of the ADC product. Additional analytical methods to those routinely used for the analysis of mAbs are required, which focus on assessing the quality attributes of ADCs that are directly related to product safety, such as DAR_{AV}, DAR profile and determination of free drug concentration.

The minimum testing protocol required for the stability testing of mAbs (Section 6, main document) **should be applied to ADCs to ensure the stability of the mAb component**, **in addition to analytical techniques that will assess the DAR_{AV}, DAR profile and free drug.**

The following is a list of those test methods which can be used to assess the quality and stability of ready-to-use ADC preparations. Where duplication with those methods listed for the analysis of mAbs occurs (main document), the limitations and/or modification which may be required for use in ADC analysis have been highlighted. The list is not exhaustive, with reports of different methods being adapted and optimised for ADC analysis regularly being published. The importance of using multiple orthogonal analytical methods for quality and stability assessment of ADCs is essential to provide confidence in the measurements.

Circular Dichroism (CD)

CD is commonly used to assess conformational changes to the secondary and tertiary structure of mAbs in the ‘near-UV’ and ‘far-UV’ regions respectively; however, this method is limited in its use in ADC analysis due to many of the cytotoxic drugs in ADCs absorbing strongly in the near-UV region causing interference and uncertainty when estimating the protein secondary structure. Far-

UV-CD can be used to assess the mAb secondary structure, providing the bound drug in the ADC does not interfere[37].

Infrared Spectroscopy (IR)

IR spectroscopy can assess chemical identity due to the unique "fingerprint" molecules have in the 1500 to 500 cm^{-1} region. Fourier-transform infrared spectroscopy has been used with attenuated total resistance sample cells to investigate the structure of proteins in liquid suspensions. It has been demonstrated that two characteristic peaks on an IR spectrum the Amide 1 and Amide 2 bands are conformationally sensitive with regards to the Alpha helix and beta sheet. Published and peer reviewed methodologies have been employed to investigate the secondary structure of multiple ADCs demonstrating neither the warheads or linkers significantly interfere with the IR signal[38].

Ultraviolet-Visible (UV/Vis) Spectroscopy

The simplest reported method for the analysis of DAR is by UV/Vis Spectroscopy, which is applied to ADCs where chromatographic methods are not suitable for accurate determination of DAR[33,38]. Due to the limitations in UV / vis spectroscopy for these products and the potential clinical consequences of misinterpretation of results this method should not be used for extended stability studies.

The UV spectra of individual peaks separated by chromatographic techniques can be used as a tool to confirm peak identifications.

Particle Counting Methods

DLS/Nanosight/RMM/MFI/LO/Microscopy can all be used to detect and quantify particulates in their relevant size ranges, as the addition of payloads does not impact the detection of aggregates or particles. However, as previously mentioned aggregation may be more of an issue with ADCs than mAbs as the payloads are hydrophobic, and in some ADCs the inter-chain disulphide bonds are broken exposing hydrophobic patches of the mAb[31,33,34].

Liquid Chromatography (LC) Methods

LC methods are the most frequently used analytical methods for characterisation of ADCs, and can provide information on the DAR profile enabling calculation of $DAR_{AV,}$ as well as molecular weight and analysis of process/storage related impurities as with regular mAbs. It should be noted that some test methods have limited use depending on the linker characteristics, for example the inherent heterogeneity of lysine conjugated ADCs limits their separation and characterisation by chromatographic applications. Example chromatograms are provided within a review of LC methods used for the characterization of ADCs[39]. A brief overview of some specific chromatographic techniques commonly employed for ADC analysis is provided in the following sections.

Hydrophobic Interaction Chromatography (HIC)

Separating sample components in order of increasing hydrophobicity, HIC is often the liquid chromatography method of choice for calculating the DAR_{av} and the DAR profile. It cannot be used to fully characterise lysine-conjugated ADCs due to the vast range of DAR and positional isomers (8.65 million possible unique variants[40]), but the data generated may be able to provide a DAR distribution fingerprint and to determine the percentage of unconjugated antibody.

In contrast to RP-HPLC, which separates proteins under harsh denaturing conditions, HIC uses mild separation conditions helping to preserve the native conformation of the ADC, thus non-covalent protein complexes such as cysteine conjugated ADCs maintain an intact mAb structure, allowing the evaluation of drug-load distribution (DOP) and calculation of DAR_{av}. Under optimal conditions, the conjugated species of the ADC are separated due to their increased hydrophobicity caused by the increased drug load, i.e. each peak corresponds to an intact mAb with an increasing number of bound drug. In the case of stochastic cysteine-linked ADCs, the unconjugated mAb (least hydrophobic) will elute first and the most hydrophobic (e.g. 8-drug conjugate) will elute last. The DAR profile can be derived from the HIC chromatogram, as the relative peak area of each peak represents the relative distribution of each drug-loaded DC species. DAR_{av} is calculated from the individual peak areas and drug load number. The disadvantages of this technique include that it is not suitable for lysine linked ADCs and the high salt content of the mobile phase makes HIC incompatible with mass spectrometry techniques.

Reverse Phase Liquid Chromatography (RPLC)

RPLC can be used to determine DAR and also unconjugated drug (free drug) content. RPLC separates proteins based on their hydrophobicity; however, whereas HIC uses mild conditions, preserving the native conformation of the ADC, RPLC uses harsh conditions that can cause denaturation of the ADC resulting in separation of non-covalently attached antibody portions, enabling the separation and quantification of heavy (H) and light (L) chains with different drug loads[38]. RPLC and HIC can therefore provide complimentary information on DAR species and positional isomers.

It is particularly useful for determining the DAR_{av} of site specifically-linked ADCs, where drugs are attached at specific amino acid residues of heavy (H), light (L) or both H and L chains; ADCs generated by partial reduction of interchain disulphides for example. Reduction of these ADCs with a reducing agent prior to RPLC analysis allows the separation of the DAR species related to the H or light L chain and therefore an assessment of the payload distribution on H and L chains. Under appropriate conditions RPLC can partially separate the positional isomers of different H species, which elute as groups of partially resolved peaks, providing information on positional isomer composition. RPLC is also compatible with MS and when used

in combination with MS detection or with information from complementary methods such as HIC and CE-SDS, information on DOP can be derived from RPLC analysis[39].

RPLC is the most commonly used analytical technique for separating and quantifying free drug; additional sample preparation steps may be required involving the precipitation of the ADC protein using organic solvent, with the free drug remaining soluble in the organic extract, or use of dual phase systems that can isolate antibody components while allowing free drug to pass through and be separated on a reverse phase column. A method to directly analyse free drug-related species in ADCs without sample preparation using a polyphenyl RP column has been described[41].

SEC

Similar to mAbs and any therapeutic protein, aggregation in ADCs has the potential to elicit an immunogenic reaction and it is critical to monitor for the presence of aggregates, especially as many of the conjugated drug species in ADCs are relatively hydrophobic and could increase the potential for aggregate formation during storage. SEC is used for separating and quantifying protein high molecular weight species (including dimers and aggregates), the monomer and low molecular weight species (including fragments). SEC methods typically used for the therapeutic mAbs may be used for the analysis of an ADC, but may require modification or adaptation due to poor peak shape and unsatisfactory resolution of aggregates from the conjugated monomer of the ADC, which are largely attributed to the hydrophobic nature of the cytotoxic drug[38]. Addition of an organic modifier to the mobile phase can successfully overcome these limitations[39]. SEC analysis using diode array detection with collection of full spectrum for the mAb and payload ranges is recommended; exploitation of these data enables confirmation that the monomer peak spectra does not change and the baseline can be monitored for drug related signals that would be indicative of small amounts of free drug.

Mass Spectrometry (MS)

MS can be used for determining DAR profile, DAR_{av} and give information on DOP if reducing or using enzymatic digests. MS works for both cysteine and lysine conjugates and is particularly useful for the characterisation of lysine conjugates given the limitations of LC methods to these molecules due to the inherent heterogeneity of lysine conjugated mAbs. Suitable liquid chromatography (LC) methods may be coupled to MS (LC-MS). Interpretation of the MS results can be challenging; deconvolution of the MS spectrum can provide information on the mass distribution that corresponds to the DAR distribution.

Capillary Electrophoresis-SDS (CE-SDS)

CE-SDS can be used for the analysis of ADCs under non-reducing and reducing conditions, and has been shown to quantify DARs that are comparable to those

obtained by analysis of the same samples using other methods, such as UV/Vis spectrometry[42], and may be useful as a complementary method to LC analysis. It should be noted that the SDS profiles of ADCs are not always expected to resemble those of a mAb, especially cysteine linked ADCs under non-reducing conditions.

icIEF

'Imaged Capillary Isoelectric Focussing' determines changes in charge state distribution of ADCs as it does with mAbs. The type of conjugation method and payload characteristic may increase the number of variants so the signal to noise ratio may be poorer for ADCs compared to mAbs; lysine conjugation with a neutral toxin linker results in increased acidic content relative to the mAb and proportional to the average number of drug molecules bound.

Bioanalytical methods

The mechanism of action of an ADC, in general terms, requires the specific binding to a target antigen via the mAb portion of the molecule, entry into the cell by internalisation (through receptor mediated endocytosis), release of the payload either by either lysosomal degradation of the mAb or cleavage of the linker (through enzymatic degradation or acid hydrolysis), allowing the cytotoxin to exert its effect by disruption of a critical cellular pathway (e.g. interference of cellular machinery such as DNA or microtubule assembly), resulting in apoptosis (programmed cell death). In addition, although the primary function of the mAb component of an ADC is to target the delivery of the drug, the mAb portion may also engage a secondary effector function such as Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) or Complement Dependent Cytotoxicity (CDC), which may contribute to the overall efficacy of the ADC.

Cell based potency/activity assays are used to measure the biological activity that a drug elicits at the cellular level by utilising an in-vitro cellular model to quantify how active or potent a molecule is by measuring the response that the cells have to the therapeutic molecule. For example, an *in vitro* cytotoxicity assay (also referred to as a cell killing assay; CKA) can be used to demonstrate the cell killing function of the ADC, and should be chosen to reflect the mechanism of action and biological function of the ADC. Examples of cytotoxicity assays that may be employed include a cell viability assay, measurement of cell apoptosis or cell cycle analysis, and will require the use of appropriate cell lines that express the targeted surface antigen. In addition, 'off-site' testing may also be employed, using a non-targeted cell line, to assess the safety of the ADC to demonstrate the viability of cells that do not express the target antigen following exposure to the ADC.

It is preferable to employ a cell-based cytotoxicity assay and this is essential where the unconjugated warhead or payload is expected to have systemic toxicity. Although binding assays (using appropriate analytical methods such as Enzyme-Linked Immunosorbent Assay (ELISA), Surface Plasmon Resonance (SPR) or

Fluorescence-Activated Cell Sorting (FACS) assay) can be employed to demonstrate the process of antigen recognition and binding of the ADC, it is important to note that binding does not demonstrate functional activity and does not necessarily correlate with DAR[43] or activity. In contrast, a cell-based cytotoxicity assay can demonstrate that the binding, internalisation and cleavage processes are retained. There may be exceptional circumstances when a binding assay will be acceptable, however, this would only be on demonstration that all physicochemical attributes of the ADC had been retained; if the results indicate any changes to the physicochemical properties of the ADC then a cytotoxicity assay must be employed to determine any biological significance of these changes. The use of other potency assays in combination with a cell-based assay may be warranted and a further understanding of antigen binding and Fc receptor binding might also be required. In any case, the method chosen to demonstrate the biological function of the ADC must be fully justified.

Cell based assay design considerations

In summary, for a cell-based assay to be effective in setting and establishing stability profiles and conditions, the assay design must consider the following:

1. The assay design must reflect the therapeutic mechanism of action (MoA) of the drug in question. For ADC's the therapeutic MoA is normally cytotoxic cell death of targeted cells via the internalised toxin, but the antibody component of ADC's may also elicit other cellular responses such as Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) and Complement Dependent Cytotoxicity (CDC). In these instances consideration must be applied into what the most significant MoA is for the therapeutic and in some instances the use of multiple cell based assays reflecting different MoAs may be required.

2. The cell line/s selected must be representative of the therapeutic activity in some way. For ADC therapeutics, the main consideration is that the cell line possesses the target surface protein/ligand/receptor of the antibody component of the ADC in sufficient quantities to elicit a detectable effect.

3. For ADC therapeutics, a cell-based assay should be designed with specificity in mind. As ADC therapeutics possess a toxin that possesses the ability to inherently kill cells non-specifically if not effectively bound to the antibody, then specificity studies should be conducted to prove that the effect elicited in the cell-based assay are in fact due to specific targeting and binding. This can be in the form of development studies that prove the molecule only binds and kills cells that possess the receptor of interest and can also include antibody and/or toxin only tests to demonstrate that there are clear observable differences in the observed response.

4. A suitable detection method must be selected that is in line with the MoA. Common methods of detection for ADCs are; cell signalling (e.g. levels of phosphorylated Akt), cell death (apoptosis – measured by flow cytometry) or cell proliferation (measure of metabolism e.g. MTT assay (or preferably using soluble formazan dyes), Cell titre Glo).

5. It is best practice to use a high number of replicates for cell-based assays to help combat inherent variation in the assay performance and to improve the robustness of the results obtained. There is normally a compromise to be made between the level of replication and the number of points within the standard curve due to limited space on 96 well plates.

6. Other considerations include the performance of the selected cell line to ensure a consistent response across the study. This requires an in depth understanding of the cell line, which is accomplished via literature reviews, trending and also keeping culture conditions as consistent as possible. Where possible Thaw and Use cell vials should be evaluated and created as they should be considered a gold standard approach for reducing cell assay variability.

7. All cell-based methods employed in stability studies should be fully validated in line with ICH guidelines[44,45].

Summary

Analytical methods used to validate stability in compounded ready-to-use ADC preparations will need to address quality attributes specific to ADCs, in addition to those applicable to all biopharmaceuticals detailed in section 6 of this document.

Of particular note are:

- Changes to the core mAb that could potentially reduce the colloidal stability, for example, the impact of disulphide bridge loss in brentuximab vedotin and increase of surface hydrophobicity in both brentuximab vedotin and trastuzumab emtansine.
- The DAR_{AV} or DAR Profile, changes to which may result in pharmacodynamic and pharmacokinetic differences.
- Increases in free drug from unintentional and premature cleavage causing potentially serious toxicity issues to patient and carer, as well as potentially reduced or increased cytotoxicity at the action site.

Handling Guidelines

Payloads are typically highly potent cytotoxic agents, up to 4000 times more potent than standard cytotoxics. In general, bound to the antibody the payload is not actively toxic, however, released from the antibody many are likely to be highly toxic although some payloads are not toxic until intracellular and cannot enter cells in an unconjugated state[28].

Linkage is susceptible to acid, base, enzymatic or nucleophilic attack and could be broken down by contact with skin proteases. The mAb and linker are also likely to be broken down by disinfectants such as hydrogen peroxide and chlorine-based agents, as well as alcohol based agents[46].

Preparation, as for cytotoxic drugs, must be restricted to pharmacy aseptic services and handling within an isolator (preferentially negative pressure). It is not appropriate to reconstitute or dilute ADC preparations on wards under any circumstances. Outsourcing of ready-to-administer ADCs may be limited by the short SmPC shelf life applied to products (routinely 4 hours to 24 hours) and relatively few stability studies having been carried out to this standard.

Some ADCs are highly light sensitive and need to be protected from light during reconstitution, dilution, storage and administration[47,48].

Cleaning / spillages

Use of harsh disinfectants such as oxidizing agents (chlorine based/hydrogen peroxide-based agents) or strong alkali solutions should be avoided for cleaning purposes following handling of ADCs, due to the risk of releasing the toxic payload. Water and neutral detergents are likely to be fairly safe[46].

Spillages must be handled as cytotoxic agent spills but again need to be dealt with without using harsh disinfectants, your spillage policy should be reviewed to ensure that it refers to ADCs and uses appropriate agents for dealing with ADC spills.

Disposal

Disposal of waste and contaminated items should be in line with other cytotoxic drugs.

Labelling

Alongside other biopharmaceuticals both the INN and the brand name should be included on the label and the products should be labelled as cytotoxic. Specific labelling as a Cytotoxic Antibody Drug Conjugate alongside standard cytotoxic labelling should also be considered.

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