

**A Standard Protocol
for Deriving and Assessment
of Stability**

**Part 2 – Aseptic Preparations
(Biopharmaceuticals)**

Edition 3

April 2017

Endorsed and supported by:



This document has been produced on behalf of the NHS Pharmaceutical Quality Assurance Committee, the NHS Pharmaceutical Production Committee and the NHS Pharmaceutical Aseptic Services Group by the NHS Pharmaceutical Research and Development Working Group. Membership of the NHS Pharmaceutical Research and Development Working Group is shown below.

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Thanks also to Lyndsay Davies, Quality Control North West (Liverpool).

Industrial, academic and regulatory experts have been consulted in the preparation of these standards.

Document History	Issue date and reason for change
Edition 1	Issued October 2012
Edition 2	Issued December 2015 – update of techniques used for analysis of 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 Obscuration and addition of reference, 11 on-going stability evaluation and introduction updated document in series
Edition 4	

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 (YCD). This document, now in its third version, is the second in a series looking at stability of pharmaceuticals 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 which are aseptically manipulated, principally monoclonal antibodies, 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 where all relevant ICH guidelines must be followed.

2. Introduction

Enabling preparation or procurement of ready-to-administer biopharmaceuticals, and in particular for monoclonal antibody (mAb), mAb fragment products or fusion proteins, is driven by both patient safety¹ and potential cost savings from more efficient product use.

Stability is particularly important with larger protein molecules because their therapeutic effects depend upon their structural integrity. Any factor causing physical or chemical instability can alter the 3D structure and folding of the protein. Monoclonal antibodies, for example, are made up of four polypeptide chains (1300 amino acid residues) with two antigen binding sites which are critical to activity. The secondary and tertiary structure may not be so critical for some small polypeptide molecules such as insulin and heparin.

Due to the intricate nature of biopharmaceuticals the situation regarding assessment of their stability is highly complex and needs specialist input in order to interpret data and design robust stability trials.

It should also be borne in mind that stability trials on biologicals and particularly monoclonal antibody products are expensive to commission and run and hence there is likely to be a reluctance from suppliers to share 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 exists 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 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 limited and generally further stability data is not forthcoming from the relevant manufacturers.

2.2 Although published studies for monoclonal antibodies are improving in quality and robustness, often published data may be of limited value to clinical need due to a restricted range of analytical techniques, restricted range or single concentrations, 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 present part of the stability picture. However, these must be independently assessed against the standards in this document.

Manufacturing processes, final product containers and all consumables must be matched to the published study 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.

2.3 Stability of biopharmaceuticals can be affected by different handling procedures and 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 raft of techniques including physical, chemical and biological assessment of activity^{2,3,4}. Shelf life cannot be determined by a single stability indicating assay but instead requires evaluation of the evidence from the whole study.

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

2.7 All Biopharmaceuticals should always be named using both their approved name and their brand name. It is not possible to extrapolate data to biosimilars or other brands to that studied.

2.8 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, the integrity of the bonding between the two components is critical for safe clinical use.

Currently this group of products is outside of the scope of this document, although the principles may be relevant in assessing the antibody component of the conjugate. A further yellow cover standards document in this series will be produced to cover this group of products once there is sufficient information and understanding of suitable stability indicating analytical techniques for these molecules.

Procurement of aseptically compounded biopharmaceuticals should only be considered where either the shelf life assigned is within the SmPC or starting material manufacturers stability data, 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 group⁵.

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 developed using one or more of the following biotechnology techniques:- recombinant DNA, controlled gene transfer / expression and 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 which are manufactured to have equivalent biological activity to an Authorised reference product. 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 decreased 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 term biogenerics has been used for these products but it is generally felt that 'biosimilar' is a better description and hence is more widely used. They are not identical to the comparator product and therefore data cannot be extrapolated from originator products to biosimilars.

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

Variability is inherent in biopharmaceuticals due to their manufacturing process; this means that stability assessment, 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 EU pharmacovigilance legislation.

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 then the higher the risks of an undetectable but clinically significant change occurring in the molecule. For this reason then studies with a short but usable shelf life assignment may often result in a better 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 miss-folding 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, excessive agitation and types of surfaces and interfaces.

Temperature enhances the rate of chemical degradation processes; it may also denature molecules which can then lead to aggregation. Freezing can also lead to 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 miss folding. All of these may lead to a conformational change causing reduced activity.

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 life⁶.

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

Contact with metals, silicone oil and other excipients can also enhance degradation, and this needs to be borne in mind when considering 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 a robust stability trial.

5. Stability Assessment

The stability of biopharmaceuticals is complex and needs to be assessed using a raft of techniques. These techniques must include stability indicating analytical methods to assure chemical and physical stability and also confirmation of biological activity using a suitable biological or biochemical assay. Where there are multiple mechanisms of action which contribute to clinical efficacy then it may be necessary to apply more than one biological cellular assay¹² 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 more stringent scrutiny when assessing its applicability to the clinical situation.

It is generally not possible to obtain reference standards for biopharmaceuticals and so the un-manipulated or freshly manipulated licensed medicine is often used as a reference.

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. If techniques selected are found to be unsuitable then the study should be abandoned and rethought.

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.

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, certainly where these containers have different characteristics (different materials, head space etc.) Studies should be carried out in the specific containers and closure systems to be used in clinical practice. The method of filling of the containers should also be clearly defined and should reflect normal practice as even this 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 a range of container sizes if applicable. However, this could prove prohibitively expensive, 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.) then it may be possible to extrapolate 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, therefore a smaller container would be considered the worst case scenario. This hypothesis cannot be extrapolated to preparations stored in syringes.

Container integrity should form an integral part of the stability assessment for all container types, 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'¹³ 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, two piece polypropylene syringes may be required for some products although past history indicates that there may be more issues with the integrity testing¹⁴.

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 compromise integrity during shipping and distribution.

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 a different manufacturers' container, there is some evidence that not all polyolefin bags behave the same with all biologicals. 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, on handling and transport.

It is not currently common practice to administer biopharmaceuticals in other container types such as 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 then accelerated conditions are generally not appropriate for stability trials. Hence all stability trials must be real time, real condition studies.

Generally, only two storage conditions are required.

- a) Refrigerated in the absence of light ($5^{\circ}\text{C} \pm 3^{\circ}\text{C}$)
- b) Room temperature ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$)

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, this would normally be limited to 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. 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 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 humidity control in the stability study.

5.4. Concentrations

Ideally each drug should be studied at a low and high clinically significant concentration. 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.

5.5. Storage Period / Study Length

Due to inherent variability in these molecules, for reconstituted products it is always appropriate 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 normal to assign a short but practical study period of between 48 hours and three months. It is not possible to extrapolate a shelf life beyond the study period. It is now expected that the study should be extended to one

time point beyond the proposed shelf life of the product, 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 holders⁶.

5.6. Sampling Strategy

It is recommended that each study includes at least four sampling points in addition to the baseline (T=0) data. These will be spread over the study period. However, if looking to assign a shelf life of six months or more then the sampling frequency must be a minimum of monthly for the first three months and three monthly thereafter as specified in the ICH Q5C³. As stated above such extended shelf life assignment is not normally recommended for biopharmaceutical products.

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.

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 then 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. The ideal is for three truly independent batches of starting material to be included in the study, as expected by ICH guidelines. This will allow assessment of stability variability related to product heterogeneity and any variations in the manufacturing process. Each of these batches should have a minimum of three replicates tested at each time point. For some assay types (see below) more replicates may be necessary. A risk based approach is acceptable and bracketing and matrixing may be used in order to carry out a cost-effective but meaningful stability study.

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 which can be carried out by weight checking following storage under ICH low humidity storage conditions).
- b) Container leachables.
- c) Additional excipient concentrations.

Note that each of these assessments may include a raft of tests 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 should 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 UV light.
- d) Agitation.

The methods used for forced degradation must be validated and must result in a detectable change, and the techniques used must be justified.

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 analysis⁷ and therefore smaller sample sizes for particle analysis may be acceptable.

Microflow Imaging may be a better option for biopharmaceuticals, this technique is more sensitive (1µm) and can differentiate sources of particles, for instance telling apart aggregates and silicone oil droplets which could be vital when looking at products in syringes. Another advantage that this technique offers 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 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 analysis of biopharmaceuticals^{8,9,10}.

a) Size Exclusion Chromatography

This is a chromatography technique where molecules are separated by their size, with different sized molecules passing through a column at different rates allowing 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 degradants cannot be characterised using this technique.

b) Dynamic Light Scattering (DLS)

This is another method of use 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. The technique measures the hydrodynamic radius as the equivalent size of a theoretical sphere.

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

Proteins are charged molecules due to the ionisable side chains and can 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 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.

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.

e) Circular Dichroism

Circular Dichroism (CD) spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light which 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.

f) 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 may have a role with some biopharmaceuticals. Often selective fragmentation has to take place ahead of HPLC analysis, detection may use a variety of techniques including 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.

c) Mass Spectrometry

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 and measuring their mass-to-charge ratio. MS can be used as an assay for the active protein and for the detection and characterisation of degradants.

There are three types of mass spectrometry of use in the analysis of biopharmaceuticals. Firstly whole mass analysis (LC-MS), which can be run on the native protein (non-reduced) or on a reduced form, this 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.

Thirdly, there is the technique of amino acid sequencing which involves proteolysis followed by LC-MS/MS, 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 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 which contribute to clinical efficacy then it may be necessary to apply more than one biological assay¹⁵. Results of biological assays should be expressed as units of activity compared with the un-manipulated or freshly compounded product.

There are three main types of biological assays.

a) Biochemical assays

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

Enzyme Linked Immuno-Sorbent Assay (ELISA) is a method of assessment of 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. This technique does not give an indication of biological response, for example glycosylated and fucosylated Rituximab have the same binding activity but different clinical efficacy.

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

b) Cell culture based assays

These 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 of the biological activity at a cellular and molecular level is of paramount importance when designing such studies.

A valid cellular process to measure must be determined, for example expressing certain receptors. An appropriate cell line then needs to be chosen which must express the relevant receptors. Due to inherent variability in cellular responses a pragmatic view has to be taken on reproducibility. Nevertheless 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 provided *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).

Although in some studies there is a good dose-response curve¹² this is not always the case. Therefore determination of the 'dose' for the biological assay can be

difficult. It is suggested that two concentrations are used; one clinically relevant, and a second that is sub-clinical.

It is usual to use a high number of replicates for cell based assays to allow for inherent variation in the 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 controls¹².

c) Animal based assays

These 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.

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 much higher than that seen for traditional physico-chemical assays, hence the biological assay data will require close scrutiny alongside the physico-chemical assay data and other factors.

Subject to the capability of the specific assay technique, maximum acceptance criteria for the physico-chemical testing should be a 5% loss in active protein (ideally much lower than this) and a maximum 2% relative to the main peak increase in any degradant peaks.

Any 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 then these molecules should be characterised where possible and their impact on biological activity, immunogenicity or other undesirable affects should be assessed.

There should be no physical change in appearance and no significant change in pH (defined as 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. Bearing in mind the inherent variability in biological assays specific acceptance criteria have not been included in this document. 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.

There is some evidence ^{11,12} that monoclonal antibodies may improve their levels of biological activity and particulate contamination levels on short term storage up to the shelf life specified in their SmPC. This fact may need to be considered when assigning acceptance criteria.

8.1 In-use storage

It is important to understand the use of the product and 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 particularly if the result is an increase in infusion time.

9. Statistical Concepts

Where appropriate statistical analysis should be applied to the quantitative data. However, the shelf life decision is likely to need to be based on a review of all the data generated from the full range of techniques used.

10. 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.

11. 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 trials¹¹. 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 is still relevant.

Pharmacovigilance reporting for biopharmaceuticals is of paramount importance and if reporting an Adverse Drug Reaction involving a biopharmaceutical then 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.

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.

References

1. NPSA Patient Safety alert 20 (28 March 2007) – Promoting Safer Use of Injectable Medicines. www.nrls.npsa.nhs.uk/resources/?entryid45=59812
2. ICH Topic Q5C / 3AB5a Quality of Biotechnological Products: Stability Testing of Biotechnological / Biological products translated via Directive 75/318/EEC www.ich.org/products/guidelines.html
3. Guideline on Development, Production, Characterisation and Specifications for Monoclonal Antibodies and Related Products – EMEA/CHMP/BWP/157653/2007
4. Test Procedures and Acceptance Criteria for Biotechnological/Biological Products Q6B www.ich.org/products/guidelines.html
5. Assessment Template (Biopharmaceuticals) – NHS Pharmaceutical R&D Group. www.qcnw.nhs.uk
6. Guidance for Specials Licence Holders, MHRA (Q&As document) version 2, January 2015
7. Pharmaceutical feasibility of sub-visible particle analysis in parenterals with reduced volume light obscuration methods, European Journal of Pharmaceutics and Biopharmaceutics 85 (2013) 1084–1087, Andrea Hawe , Frank Schaubhut , Raimund Geidobler, Michael Wiggenghorn, Wolfgang Friess, Markus Rast, Christian de Muynck , Gerhard Winter
8. USP 39 Published General Chapter <129> Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies
9. Proposed USP General Chapter <507> Protein Determination Procedures
10. Proposed USP General Chapter <212> Oligosaccharide Analysis
11. Monoclonal Antibodies – Functional Improvements at SPC Shelf-Life Limits. R.V. Parry, T. Chapman, M. Ali Khan, M. Connolly, A.G Watts presented at BOPA 2014.
12. Evaluation of the physicochemical and functional stability of diluted REMSIMA® upon extended storage - A study compliant with NHS (UK) guidance, B.L. Young, M.A. Khan, T.J. Chapman, R. Parry, M.A. Connolly, A.G. Watts, Int. J. of Pharmaceutics 496, December 2015, 421– 431 <http://www.sciencedirect.com/science/article/pii/S0378517315302842>
13. Protocol for the integrity testing of syringes - NHS PQA Committee. www.qcnw.nhs.uk.
14. National collated data from syringe integrity testing – NHS Pharmaceutical R&D Group. www.qcnw.nhs.uk.
15. ICH Q5E Comparability of Biotechnological / Biological products subject to Changes in their Manufacturing process www.ich.org/products/guidelines.html

Appendix 1

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

The following checklist is provided as a quick guide to assessing 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 this 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 it 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?	

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

Approved:.....Date:.....

Additional risk reduction measures