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REVIEW

Analytical comparability study of recombinant monoclonal antibody therapeutics

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ABSTRACT

Process changes are inevitable in the life cycle of recombinant monoclonal antibody therapeutics. Products made using pre- and post-change processes are required to be comparable as demonstrated by comparability studies to qualify for continuous development and commercial supply. Establishment of comparability is a systematic process of gathering and evaluating data based on scientific understanding and clinical experience of the relationship between product quality attributes and their impact on safety and efficacy. This review summarizes the current understanding of various modifications of recombinant monoclonal antibodies. It further outlines the critical steps in designing and executing successful comparability studies to support process changes at different stages of a product's lifecycle.

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Process changes are inevitable in the life cycle of recombinant monoclonal antibody therapeutics. Products made using pre- and post-change processes are required to be comparable as demonstrated by comparability studies to qualify for continuous development and commercial supply. Establishment of comparability is a systematic process of gathering and evaluating data based on scientific understanding and clinical experience of the relationship between product quality attributes and their impact on safety and efficacy. Here, we summarize the current understanding of various modifications of recombinant monoclonal antibodies, and outline the critical steps in designing and executing successful comparability studies to support process changes at different stages of a product's lifecycle.

The lifecycle of recombinant monoclonal antibody (mAb) therapeutics extends from early through late development stages and even after marketing approval, and process changes are highly likely to occur over the course of this long period. During early development, it is common practice for pharmaceutical companies to focus on rapid advancement to first-in-human studies in order to achieve proof-of-concept while gaining knowledge to inform subsequent development decisions. Continued process optimization is therefore necessary to meet regulatory requirements toward late-stage development, and to have a robust process heading into commercial manufacturing. This approach helps companies allocate resources appropriately based on the development risk and potential commercial success. Ultimately, the aim is to help patients gain rapid access to affordable therapeutics based on the latest scientific breakthroughs. Changes are also often made to a commercial process for a variety of reasons, such as adapting to evolving regulatory requirements; meeting additional market demand; implementing newer techniques for higher product yield and better quality; or addressing a gap due to discontinuation of equipment, chromatography resins, raw materials, or commercial manufacturing sites. These changes can all potentially lead to a product with significantly different properties from the pre-change product, thereby affecting the quality of the pharmaceutical product. For the foregoing reasons, such changes require evaluation by comparability studies to ensure that post-change products are of comparable quality to the pre-change products with respect to structural characteristics, biological functions, and stability, which, in turn, provide assurance of consistent product identity, safety and efficacy.

As the goal of a comparability study is to demonstrate that the pre-change and post-change products are comparable, the results can validate the use of safety and efficacy data generated using pre-change material to support the next phase of development or continuous commercial supply. The earliest time that the comparability exercise applies is between the nonclinical materials used for investigational new drug (IND) application-enabling studies and Phase 1 clinical material. Comprehensive comparability studies, including a thorough evaluation of the product quality using data from routine lot release; extended characterization, including isolation and characterization of variants and impurities; in-

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process testing, stability and forced degradation, are performed for late stage comparability studies. Depending on the extent of the change, comparability can be demonstrated solely by analytical data, including biochemical, biophysical and functional data. However, if analytical comparability is not established, nonclinical and clinical studies will be required. Establishing comparability based on strong analytical data alone, without going through nonclinical and clinical evaluations, is of mutual benefit for patients and companies because it saves resources and accelerates development. Health authorities encourage sponsors to discuss process changes and comparability studies to ensure that the sponsors' comparability strategy and regulatory expectations are aligned for seamless product development.

Scientific understanding of quality attributes and their relationship to safety and efficacy plays an essential role during comparability evaluation. Understanding the quality attributes helps predict the impact of process changes on product quality and enables knowledge-driven risk assessment. A thorough understanding of the critical quality attributes (CQAs) helps in the design of comparability studies that focus on attributes that are likely affected by the process changes and those that have the potential to affect safety and efficacy. Such fundamental scientific understanding is the foundation of risk assessment when certain attributes are outside the historical trend of pre-defined acceptance criteria.

Here, we discuss the most common quality attributes detected in recombinant mAbs and their impact on structure, function, stability and pharmaceutical properties. An adequate level of understanding of the structure-function relationship provides the scientific rational for establishing comparability. Our review also covers, in detail, the current International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and other relevant regulatory guidance, including those from the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), and provides practical considerations for the design and execution of phase-appropriate comparability studies.

1. Current understanding of recombinant monoclonal antibodies

Recombinant mAbs are glycoproteins of approximately 150 kDa with a high level of heterogeneity due to various post-translational modifications (PTMs) and degradation events that occur at all stages of the manufacturing process.[1–4] Most mAbs are produced using mammalian cell lines, such as Chinese hamster ovary (CHO) or murine cell lines (e.g., NS0 or SP2/0). The complexity, structure-activity relationship, and relationship between quality attributes and safety and efficacy should be taken into consideration for comparability studies, as recommended in a number of guideline documents.[5–9] General knowledge of mAbs and a specific understanding of the molecule in development obtained via analytical characterization are critical for a successful comparability study.

The currently known PTMs and mAb degradation products that are generated during manufacturing and storage are summarized in Table 1, and, in the section below, each attribute is

Table 1. Common PTMs of mAbs and their potential effects on stability, function, immunogenicity and pharmacokinetics/pharmacodynamics.

Attributes	Potential impact
N-terminal modifications • PyroGlu • Leader sequence • Truncation	N-terminal modifications generate charge variants. They are considered to be low risk to comparability because of lack of impact on efficacy and are not expected to impact safety.
C-terminal modifications • Partial removal of C-terminal lysine • Amidation • Truncation	C-terminal modifications generate charge variants. They are considered to be low risk to comparability because of their low percentage and lack of impact on efficacy and are not expected to impact safety.
Fc-glycosylation • Sialic acid • α-1,3 Gal • Terminal Gal • Absence of core-fucosylation • High mannose	 • N-Glycolylneuraminic acid (NGNA) is immunogenic • α-1,3 Gal on Fab oligosaccharides is immunogenic • The presence of galactose enhances complement-dependent cytotoxicity (CDC) and possible shorter half-life if exposed • The absence of core-fucose enhances antibody-dependent cell mediated cytotoxicity (ADCC) • mAbs with high mannose show enhanced ADCC and shorter half-life
Asn deamidation	Deamidation in complementarity-determining region (CDR) can potentially decrease potency
Asp isomerization	Isomerization in CDR can potentially decrease potency
Succinimide	Succinimide in CDR can potentially decrease potency
Met and Trp Oxidation	Oxidation in CDR can potentially decrease potency. A substantial amount of oxidation around FcRn binding site can potentially decrease its binding affinity and result a in shorter half-life
Cysteine-related variants • Disulfide isoforms • Free cysteine • Trisulfide bond • Thioether, D-cysteine, cysteinylation	IgG2 disulfide bond isoforms may impact potency. Higher amounts of free cysteines decrease mAb thermal stability and trigger formation of covalent aggregates. Other modifications such as thioether, D-cysteine, and cysteinylation are considered low risk because of their low levels or natural presence in humans
Glycation	Glycation is a common modification leading to the generation of acidic species. Glycation in CDRs can potentially decrease potency. Glycation in general increases mAb propensity towards aggregation
Fragments	Fragments are considered as low risk because of their low levels.
Aggregates	Aggregation can potentially cause immunogenicity and loss of efficacy. It is a high risk factor for comparability.

discussed in detail with regard to the chemical nature, and its impact on structure, stability, efficacy and *in vivo* half-life.

1.1. N-terminal modifications

The two most common N-terminal modifications of a mAb are the presence of pyroglutamate (pyroGlu) as the first amino acids of the mature light chain or the heavy chain and the presence of unprocessed leader sequences.

PyroGlu is formed via the spontaneous cyclization of glutamine (Gln) and glutamate (Glu) side chain onto the N-terminal alpha amine. Formation of a five-member ring is thermodynamically favored and occurs readily for Gln.[10–13] This cyclization is also favored at neutral and slightly basic pH at elevated temperatures typically encountered during cell culture.[10] N-terminal glutamate can also be cyclized to form pyroGlu, but this occurs at a much slower rate.[14,15]

MAb transgenes incorporated in the genome of the host organisms typically contain a 10–15 amino acid leader sequence responsible for guiding the protein through the secretion machinery. In most cases, the leader sequence on the light chain and heavy chain is removed via proteolysis upon secretion of the recombinant mAbs into the cell culture broth. However, in a number of instances, the leader sequence is not completely processed, resulting in antibodies with an entire or a partial leader sequence.[11,16] In rare cases, miscleavages result in truncation.[17,18]

The presence of N-terminal pyroGlu or a leader sequence is not expected to affect the overall structure and function of recombinant mAbs The presence of N-terminal glutamine or pyroGlu has no impact on potency.[11] In human endogenous immunoglobulin (IgG), the N-terminal glutamine is almost completely converted into pyroGlu,[19] minimizing immunogenicity concerns for this modification. However, both modifications introduce charge differences to the molecules, which may affect inter-molecular interaction, and thus potentially cause aggregation. Miscleavage of the signal peptide resulting in N-terminal elongation or truncation is undesirable for mAb manufacturing because it causes product heterogeneity. The unprocessed or partially processed leader sequence, which is hydrophobic in nature, may facilitate the formation of aggregates and remains an unknown factor to immunogenicity.

1.2. C-terminal modification

Removal of C-terminal lysine (Lys) and C-terminal amidation are the two major C-terminal modifications. Human gamma heavy chain IgG genes contain a terminal codon translated as a lysine residue. However, human IgG circulating in the bloodstream does not contain the terminal lysine because it is removed enzymatically.[20] It is customary for transgenes of recombinant mAbs to also include a terminal lysine codon. The C-terminal lysine is typically enzymatically removed during cell culture.[21] Depending on cell culture conditions, removal of C-terminal Lys can be incomplete, resulting in antibodies with either zero, one or two C-terminal Lys residues. Lys residues carry positive charges, so antibodies with either one or two C-terminal Lys residues can easily be detected by charge-based methods as basic species.[12]

Amidation of the antepenultimate residue at the heavy chain C-terminus is another common modification of recombinant mAbs.[22–24] The product of C-terminal amidation is removal of the newly exposed glycine and amidation of the proline residue in the case of IgG1 and of the leucine residue for IgG4.[23] Because IgG2s and IgG3s have the same C-terminal amino acids as IgG1s, amidation is also likely to occur at the heavy chain C-termini of these two subclasses. C-terminal alpha amidation can also be detected as a basic variant by charge-based methods.[22]

Because C-terminal Lys and C-terminal amidation modifications are at the extremities of mAbs and remote from the antigen, neonatal Fc receptor (FcRn), and Fc gamma receptor binding sites, these modifications are not expected to affect antibody structure, stability and function. No structural differences were observed between antibody fractions with either zero, one or two C-terminal lysine residues by hydrogen-deuterium exchange[25] or differential scanning calorimetry.[26] It has also been demonstrated that the C-terminal Lys has no impact on antigen binding.[11,27] Antes et al demonstrated that the C-terminal Lys has no impact on potency mediated through complement activation.[27] However, a more recent study demonstrated that removal of the C-terminal Lys is required for optimal C1q binding and complement-dependent cytotoxicity (CDC).[28] C-terminal Lys can be rapidly removed in circulation in humans.[20] In a detailed study with mutants lacking C-terminal Lys, or lacking both C-terminal Lys residues and the preceding glycine (Gly), it was found that the C-terminal Lys has no impact on potency, bio-availability, and pharmacokinetics (PK); however, depending on the specific mAb, it may decrease thermal stability.[29] Interestingly, antibodies expressed from constructs lacking the C-terminal Lys codon showed decreased expression titer due to slower synthesis and faster degradation rates.[30] Due to complete in vivo processing, human IgG are present without the C-terminal lysine.[20] As is the case with C-terminal lysine, C-terminal amidation has no effect on antigen binding and Fc functions.[22] Although IgG C-terminal amidation has not been reported in human IgG,[23] it is commonly detected in hormone peptides.[31] From this body of knowledge, it can be concluded that neither C-terminal Lys nor amidation is expected to impact mAb structure, stability, function or safety. Practically, product efficacy and safety should not be affected if characterization of the molecule indicates that the charge difference observed between pre- and post- change lots is solely due to the level of C-terminal Lys.[32]

1.3. Fc glycosylation

Glycosylation of the conserved asparagine (Asn) of the heavy chain constant domain 2 (CH_2) is important for maintaining mAb structure and stability[33–39] and, in certain cases, modulating Fc-mediated biological functions.[36,39–42] MAbs produced in CHO cell lines have mainly biantennary structures similar to those found in human endogenous IgGs, while mAbs expressed in murine cell lines such as NS0 cell lines contain non-human glycan moieties, such as N-glycolylneuraminic acid (NGNA) and α-galactose. Nevertheless, N-glycosylation is one of the main contributors to the heterogeneity of mAbs, with the majority of variations arising from different levels of sialylation,

galactosylation, fucosylation and high mannose type of oligosaccharides. In rare cases, recombinant mAbs may also have N-glycosylation in the antigen-binding fragments (Fabs), and these N-glycan structures are often heavily sialylated.[43] N-glycosylation is one of the most sensitive indicators of manufacturing consistency, and therefore is of particular interest for comparability studies.

1.3.1. Sialic acid

The type of sialic acid present in human endogenous IgG is N-acetylneuraminic acid (NANA)[44,45] attached to terminal galactose via an α-2,3 or α-2,6 linkage. MAbs expressed in CHO cell lines contain NANA, but in the α-2,3 linkage only. The CHO genome encodes the gene for cytidine monophosphate-N-acetylneuraminic acid hydroxylase (Cmah), which is the enzyme that catalyzes hydroxylation of NANA to form NGNA, but Cmah is not expressed under normal conditions[46] resulting in the absence of the immunogenic non-human NGNA in mAbs.[47] In contrast to mAbs expressed in CHO, mAbs expressed in NS0 and SP2/0 cell lines contain NGNA.[44,45]

Sialic acids on the conserved Fc-glycans of mAbs are present at levels rarely exceeding 5%.[43,45,48] In rare cases where N-linked oligosaccharides are also present in the Fab region, relatively higher amounts of sialic acid have been observed.[43] The level of sialic acid in human endogenous IgG is ~ 11%-15%,[44,45,49] due to the presence of N-linked oligosaccharides in the Fab regions. Studies to date indicate that the presence of such levels of Fc-associated sialic acid only results in a subtle conformational difference[50-55] when compared with their asialylated counterparts. The presence of Fc sialic acid does not cause differences in aggregation propensity under thermal stress,[56] and has no effect on antigen binding[40,57,58] or half-life.[57–60] The effect of sialic acid on receptor binding is inconclusive because several studies demonstrated that sialic acid decreases antibody binding to FcΓ receptors and related biological activity,[57,58,61] whereas other studies demonstrated that sialic acid has no effect on FcγR1 binding, antibody-dependent cell-mediated cytotoxicity (ADCC) and CDC.[40,60,62]

In general, the presence of NGNA is not a major concern because of its low levels, especially in CHO-produced mAbs. However, it is important to closely monitor and control the level of NGNA and carefully evaluate the levels when comparing pre- and post-change lots during comparability studies.

1.3.2. Terminal galactose

Biantennary complex oligosaccharides in the Fc region with either zero, one or two terminal galactose are the three major glycoforms in mAbs.[45,48] A similar galactosylation pattern is found in human endogenous IgG.[44,49] The degree of galactosylation only causes a subtle local conformational change[33,36,41,63,64] and does not affect the propensity to form aggregates under thermal stress.[37,56] Galactosylation of the Fc glycans has no impact on in vivo clearance.[59,60,65–68] In contrast, exposed Fab glycans with a high degree of galactosylation, uncapped by sialic acid, may result in a significant reduction of half-life through binding to asialoglycoprotein receptors.[69]

Much attention has been devoted to the study of the impact of terminal galactosylation on biological functions, including ADCC and CDC. These studies have demonstrated either no correlation[36,40,60,62,63,70–75] or a positive[41,64,73,76–78] correlation between terminal galactose with Fcγ receptor bindings and resulting ADCC. One detailed study demonstrated that the effect depends on the types of receptors involved, with the removal of galactose from IgG1 and IgG2b decreasing their binding to FcγRII, but increasing binding to FcγRIII.[73] The impact of the degree of galactosylation of the Fc glycans on mAb binding to C1q and CDC activity has also been a subject of debate because studies reported either no correlation[36,60,62] or a positive correlation.[40,71,73,74,77] When the different studies are considered as a whole, and taking into account the more recent work, it is reasonable to conclude that galactosylation of the Fc-glycan has a limited effect on ADCC activity, but some effect on CDC.[71,79] While these conclusions primarily apply to IgG1, much less is known for mAbs of other subclasses. Therefore, the effects of galactosylation on ADCC and CDC need to be assessed on a case-by-case basis, where applicable.

Concerning galactosylation, it is important to note that some mAbs may contain α-1,3 galactose, which is potentially immunogenic.[80,81] MAbs produced in murine cell lines in particular have been shown to contain a relatively higher level of this moiety compared to mAbs manufactured using CHO cell lines.[45,82] The CHO genome contains the gene for a functional α-1-3 galactose transferase, but the gene product is only expressed in rare situations.[46,83] Nevertheless, the presence of the α-1,3 galactose antigen may only be a safety concern in the case of Fab N-glycosylation as anti-α- 1,3 galactose antibodies do not bind to the antigen when associated with Fc glycans.[84]

1.3.3. Fucose

Complex oligosaccharides with core-fucosylation of the N-acetylglucosamine (GlcNAc) attached to the protein via α-1,6 linkage is the dominant form in human IgG molecules.[49,85] The same is true for mAbs expressed in mammalian cell lines, where complex oligosaccharides without the core fucose are present only at a low levels. High mannose oligosaccharides that also lack the core-fucose are detected at low levels as well.

Variation of the fucosylation level only causes subtle differences around the glycosylation site[41,86-88] and has a minimal impact on binding of antibodies to FcγR1, FcγRII, c1q, and FcRn.[89,90] In contrast to other receptors, low core-fucosylation results in a dramatic improvement in antibody binding to FcγRIIIa[89-92] and leads to higher ADCC activity.[70,89–91] The correlation between low core-fucosylation and higher ADCC was found to translate into higher efficacy in animal disease models[91,93] as well as in human subjects for mAbs that rely on this mechanism of action (MOA).[94,95] The impact of core-fucose on antibody half-life remains unclear. Two studies performed in mice show contradictory outcomes: either faster clearance[91] or no difference.[96] Analysis of the clinical trial results of obinuzumab, which is an afucosylated glycoengineered mAb, showed steady state PK parameter values typical of mAbs.[97]

Because of the impact of core-fucose on ADCC, its level should be closely monitored for effector functions, particularly when ADCC is involved in MOA or for antibodies that target cell surface antigens.[98] This is of particular importance for comparability assessments because of the potential impact of core-fucose differences on safety or efficacy.

1.3.4. High mannose

High-mannose N-glycans typically contain five to nine unsubstituted mannose attached to the core GlcNAc. While high mannose is present at only around 0.1% in human endogenous IgG,[49] the level can reach up to 10% in mAbs.[45,48]

Comparison of antibodies with either high mannose or complex oligosaccharides shows that their presence results in a subtle conformational change around the glycosylation site.[53,54,88] MAbs with high mannose showed decreased thermal stability,[88] with no impact on long term stability[88] or on the propensity towards aggregation when subject to thermal stress.[56] As in the case of other types of glycans, high mannose has no effect on antigen binding,[99,100] but the presence of high mannose structures resulted in reduced binding to FcγR1,[60,99] FcγRII,[101] and deficiency in C1q binding and complement activation.[60,96,99–101] MAbs with high mannose structures demonstrated increased binding to FcγRIIIA leading to enhanced ADCC[96,100,101]; however, such an impact is not due to the presence of high mannose, but rather due to lack of the core fucose.

Heightened concern around high mannose structures is related to studies indicating their effect on the PK properties of recombinant mAbs. Such studies have demonstrated that the presence of high mannose resulted in shorter *in vivo* half-life in animal models as well as in humans.[60,65,66,96,99–101] It is therefore important that, for comparability studies, the level of high mannose structures should be carefully evaluated.

1.3.5. Aglycosylation

While virtually non-existent in human endogenous IgGs, aglycosylation occurs at low but persistent levels in mAbs.[102–105] Aglycosylated IgG1 antibodies show substantial conformational differences, decreased stability and almost complete loss of the Fc effector-triggered biological functions such as ADCC and CDC.[33,39,106,107] The absence of effector functions for therapeutics where the MOA is only dependent on antigen blocking eliminates safety concerns (e.g., atezolizumab).[98] Aglycosylated IgGs are more homogeneous than canonical IgGs. Although the impact of aglycosylation on *in vivo* half-life is inconclusive in animal studies,[34,39,108] results from human clinical trials revealed a normal half-life for aglycosylated antibodies compared with their respective glycosylated molecules.[106]

The presence of aglycosylated mAbs is not expected to have a substantial impact on product quality because of their extremely low levels, and thus it may be an area of less concern from the standpoint of comparability assessment.

1.4. Deamidation

Asparagine (Asn) deamidation is perhaps one of the most prevalent PTMs in mAbs. Asn deamidation is a spontaneous reaction facilitated by neutral to basic pH and elevated temperature, typically leading to the formation of a 3-to-1 ratio of isoaspartate (isoAsp) to aspartate (Asp).[109] Cell culture, certain purification steps, accelerated stability studies and post administration by injection to animals or human subjects are conditions that favor deamidation.

Deamidation has been reported to occur in antibody complementarity-determining regions (CDRs), and resulted in decreased antigen binding affinity.[110–113] However, deamidation has been reported more frequently in the constant domains of recombinant mAbs.[11,114–117] Every effort should be made to carefully craft mAbs through protein engineering and manufacturability assessment studies so as to eliminate the risk of deamidation in the CDR, and thus alleviate any potential deleterious impact. Deamidation in the constant domains may not be avoidable, and thus should be closely monitored and controlled.

The effect of deamidation varies depending on the location of the Asn residue and the resulting products. Formation of both Asp and isoAsp introduces negative charges, which may affect the structure and stability of mAbs. In addition to directly affecting the charge of the molecule, formation of isoAsp may result in a larger structural change compared to Asp due to the addition of a methylene group to the peptide backbone. For instance, one study shows that Asn deamidation to form Asp increased Fab thermal stability, while formation of isoAsp decreased thermal stability.[112]

Besides loss of potency, another concern associated with the presence of deamidated products, especially isoAsp, is immunogenicity, as has been shown in non-mAb proteins.[118] Although the risks associated with Asn deamidation may be mitigated by adequate manufacturing controls and formulation conditions, the reaction continues to occur to mAb therapeutics during circulation.[111,119] Asn deamidation is ubiquitous *in vivo*, as evidenced by its detection in human endogenous IgG.[119] Given the widespread occurrence of isoAsp *in vivo* and its potential deleterious impact on a proteins' structure and function, it is not surprising that organisms have evolved repair mechanisms involving protein isoaspartate methyltransferase, which converts isoAsp back to Asp.[120]

1.5. Asp isomerization

Asp isomerization to form isoAsp and Asp follows the same mechanism as that of Asn deamidation, and as such is affected by the same structural constraints and environmental factors such as pH and temperature. The formation of isoAsp introduces an additional methylene group into the peptide backbone, so structural changes can be expected. Asp isomerization has been reported in several mAbs.[121–124] In a number of cases, Asp to isoAsp isomerization has a destabilizing effect on the Fab.[112] Isomerization of Asp residue in the CDR, or in close proximity to the CDR, to isoAsp has been shown to cause a decrease in potency.[110,121,122]

1.6. Succinimide formation

Succinimide is the thermodynamically favored five-member ring reaction intermediate of both Asp isomerization[121,123,125,126] and Asn deamidation.[113,127] The succinimide group is typically most stable at pH 5.0- to 6.0 at 2–8°C, but it is readily hydrolyzed at the higher pH and temperatures conditions prevalent *in vivo*.[127] The presence of succinimide in the CDRs has been shown to decrease mAb antigen binding affinity and potency.[113,121,126,127] Deamidated Asn residues are found in endogenous human IgG,[119] so the succinimide intermediate should also be expected to be present, perhaps as transient species, in IgGs and other human proteins.

1.7. Oxidation

Although, in general, several amino acid residues are susceptible to oxidation, the most prevalent oxidation events of mAbs occur to methionine[128–131] and tryptophan[132–134] residues.

Two methionine (Met) residues, conserved in all human IgG close to the CH_2-CH_3 domain interface, and also part of FcRn and Protein A and Protein G binding sites, have been shown to be consistently susceptible to oxidation.[130,135,136] Oxidation of those Met residues caused structural changes mainly in the CH_2 domain, decreased its thermal stability[130,135,136] and increased propensity towards aggregation.[135] Although there is no impact on Fcγ receptors,[128] oxidation of those residues resulted in decreased CDC activity,[130] binding affinity to FcRn[128,130,131] and to *in vivo* half-life.[137]

Tryptophan residues tend to be part of the hydrophobic core of mAbs, and thus are less exposed to solvent, except when they are located in the CDRs. Oxidation of a tryptophan residue in the CDR was shown to reduce thermal stability, increase propensity towards aggregation and reduced antigen binding and potency.[133,134] Tryptophan oxidation may also cause a yellow coloration,[138] mainly due to the formation of kynurenine.

1.8. Modifications related to cysteine residues

Modifications associated with cysteines (Cys) appear in three forms. The first is incomplete formation of disulfide bonds leading to the formation of a low level of free cysteines. The second is the formation of a non-canonical disulfide bond linkage. The third is disulfide bond degradation, which forms various unwanted products.

Although most cysteine residues are involved in the formation of disulfide bonds, low levels of free cysteine have been detected in mAbs due to either incomplete formation or reduction of formed disulfide bonds.[139–142] Cysteines located in the heavy chain variable domains seem to be more likely to exist in the free thiol form,[13,143,144] impacting antigen binding affinity.[145] The presence of low-level free cysteine resulted in decreased thermal stability.[146] Free cysteines can trigger the formation of reducible covalent aggregation due to disulfide bond scrambling.[141] These free cysteines can also react with other free cysteines present in the cell culture media to form cysteinylated adducts.[147–150] Cysteinylation of an extra cysteine residue causes conformational change, decreased thermal stability, increased propensity towards aggregation and decreased activity.[147] Free cysteines are also detected in human endogenous IgG molecules.[141,146]

Different disulfide bond-related isoforms have been reported, mainly in association with IgG2 and IgG4. In the case of IgG2s, in addition to the classical A form, two additional forms with alternative inter chain disulfide bond linkages, known as A/B and B forms, have been described.[151] IgG2A displays higher potency compared to IgG2B in a subset of molecules.[152] Different disulfide bond isoforms do not represent a concern because human endogenous IgG2 also exists as multiple disulfide bond isoforms,[151,152] and because the initially synthesized IgG2A form was shown to convert through A/B forms into IgG2B in circulation.[153]

It has long been known, prior to the discovery of the IgG2 isoforms, that, for IgG4, the two inter-heavy chain and intra-chain disulfide bonds exist in equilibrium leading to the formation of half-molecules and bispecific molecules, a phenomenon referred to as Fab arm exchange.[154,155] Bispecific molecules with each arm recognizing a different antigen have been demonstrated to occur *in vivo* to recombinant antibody and endogenous IgG4 molecules.[156,157] Natalizumab, the first commercialized IgG4, was shown to undergo Fab arm exchange *in vivo*.[158] More recently developed IgG4 molecules, including commercial products (except for reslizumab) and those in the pipeline, have the hinge region S228P point mutation to stabilize the hinge region disulfide bond, thus preventing Fab arm exchange with endogenous human IgG4 from occurring.[159]

Disulfide bonds have been demonstrated to degrade through various mechanisms depending on environmental conditions. The formed disulfide bond can be reduced during the cell culture harvesting process.[160–162] Disulfide bonds can be degraded through the β-elimination mechanism, leading to the formation of a transient sulfide ion, a mAb-associated free thiol group and a dehydroalanine side chain. Due to their relatively strong nucleophilicity, free thiols in mAbs can react with the dehydroalanine in the vicinity to form non-reducible thioether.[163,164] In the course of the preceding reaction, one of the two cysteine chiral centers becomes an enantiomeric mixture of D- and L-cysteines.[165,166] β-elimination is accelerated at basic pH and high temperature.[166] This entire process is believed to occur naturally in the human body because thioether and D-cysteine have also been detected in endogenous IgG.[164]

Another type of disulfide bond degradation leads to the formation of a trisulfide bond, which was initially identified in a recombinant monoclonal IgG2 antibody.[167,168] Prior to the formation of a trisulfide bond, the disulfide bond is most likely reduced and then re-oxidized with the addition of a sulfide atom. Trisulfide bonds have no effect on antibody thermal stability and potency.[167–169] The trisulfide bond can be rapidly converted into a disulfide bonds *in vivo*.[167] It is worth noting that trisulfide bonds have also been detected in human endogenous IgG,[164,167] perhaps indicating that such modifications, at low levels, may not have deleterious effects on product quality. From the comparability assessment perspective, such modifications should be of no concern if they occur at similar levels in the pre- and post-change materials and at levels consistent with historical data.

1.9. Glycation

Glycation is a non-enzymatic reaction between a reducing sugar and the primary amine of a lysine side chain or the N-terminal amine group of the protein. It occurs under physiological conditions and has been commonly observed during cell culture,[170,171] accelerated stability,[172] storage[173,174] and administration.[173] Glycation continues to occur to the recombinant mAbs, as well as endogenous IgG, during circulation.[175]

Glycation of lysine residues has not been shown to affect potency[170,176,177] and PK.[176] However its impact could be molecule-dependent and glycation site-dependent (e.g., whether it is located within the CDRs or not). As a result, a case-by-case study is warranted to assess the effects of glycation on potency and PK.

In addition, a study has shown that glycation accelerates aggregation.[172] Advanced glycation end products result in product coloration.[178] The level of glycation can be sufficiently controlled by modulating sugar concentrations in cell culture medium or through an appropriate feeding strategy.[170] Glycation levels should be closely monitored during storage if sugars are included in formulation. The complexity of the cell culture conditions may be such that glycation may not be completely avoided, but this is not usually the case with drug product manufacture. Glycation can be strictly controlled and even avoided for the drug product through the judicious selection of excipients.

1.10. Other chemical modifications

In addition to the afore-mentioned common modifications, a number of other types of modifications can occur, albeit occasionally. Examples include carbonylation of threonine (Thr), arginine (Arg) and Lys,[179] modification of the N-termini of the light chain or heavy chain by citric acid[180] or modification of N-termini and Lys side chain by citric acid photo degradation products.[181] During cell culture, metabolism by-product methylglyoxal can react with Arg residues,[182] while vitamin C degradation products can react with N-terminal and Lys side chains.[183] Long-term storage or exposure to lights has been shown to cause histidine (His) oxidation.[184–186] Further reactions between the oxidized His residues with other residues, including His, Cys, Lys, can result in the generation of aggregates.[185,186] Interestingly, many of the modifications result in the generation of acidic species because they are either on the side chains of lysine or arginine residues or the light chain and heavy chain N-terminal primary amine groups. The reaction products are undesirable in all these cases and need to be evaluated as part of the comparability assessment. Significant differences in the levels of these undesirable products, if present, in the pre- and post-change materials may be indicative of problematic process or product differences.

1.11. Fragmentation

Non-enzymatic mediated fragmentation is common in mAbs,[103,115,187–197] occurring around the hinge region,[189,191,192,195,197–200] at the domain-domain interface,[195] as well as in the CDR.[124,201] Other than direct hydrolysis, fragmentation has also been reported to be catalyzed by metals such as copper[200] and iron[202] or by such nonmetallic substances as H_2O_2.[203]

In general, fragmentation in mAbs is not a major concern. Fragmentation is sufficiently controlled due to the fact that most formulations employ mild pH, and the drug substance and drug product are stored under freezing or refrigerated conditions that do not promote fragmentation. It is therefore not expected that fragmentation will be a major area of interest during the comparability assessment of pre- and post-change materials.

1.12. Aggregation

Aggregates are one of the major impurities in mAb therapeutics, and are classified, by default, as a CQA because of their potential for being immunogenic. The mechanism leading to aggregation is complex and likely to be idiosyncratic to each mAb. Nevertheless, it is commonly believed that aggregation may begin upon local unfolding and exposure of hydrophobic patches.[204] Attempts have also been made to identify and eliminate regions in mAbs that are suspected of mediating aggregation.[204] Aggregation has been shown to be caused by one or the combination of different stress conditions, such as elevated temperature,[115,172,187,193,202,205–212] freeze-thaw,[196,207,210,212,213] agitation,[202,208,214–218] low pH,[193,196,219,220] high pH[193,208] and light exposure.[221,222]

The major concerns with aggregation are loss of efficacy, receptor activation through cross-linking and, most importantly, immunogenicity.[223] Although the mechanisms leading to aggregation-mediated immunogenicity are not fully elucidated, the existence of at least two parallel pathways leading to anti-drug antibodies (ADA) has been proposed. These pathways include classical T cell-dependent activation of the immune system involving antigen presenting cells and, alternatively, a T cell- independent breakdown of B cell tolerance. In the former case, neo-epitopes or the presence of elevated amounts of immunogenic PTMs in the aggregates would facilitate the mounting of an immune response. For T cell-independent B cell activation, aggregates would need to form protein clusters containing well-ordered repetitive structures that mimic the surface of pathogens to trigger an immune response.[223] However, most mAbs form amorphous high molecular weight species that are generally not associated with the breakdown of B cell tolerance. It is important to note that, even if the aggregates induce ADAs through this pathway, the ADAs generated in those situations are low affinity IgM and IgG responses without formation of B cell memory or affinity maturation. This implies that upon discontinuation of the mAb treatment, the immune reaction would naturally disappear. While ADA responses against therapeutic mAbs are not infrequent, no clinical evidence linking mAb aggregates to immunogenicity has been established to date. Nevertheless, because of the strong potential for immunogenicity and the absence of reliable predictive models to prove otherwise,[223,224] it is appropriate to take the conservative stance that aggregates pose safety risks to the patients. The potential impact of aggregates on patient safety is such that major differences in the nature and levels of these species in pre- and post-change materials would render them not comparable and could lead to a requirement for additional clinical studies to support the post-change material.[225]

1.13. Coloration

Coloration of mAb drug substances is a common quality attribute, especially for high concentration solutions. Oxidation of tryptophan residues,[138,226] the presence of advanced glycation end products (AGEs),[178] and association of mAb with B vitamins, their degradation products or B-vitamin-mediated reaction products[226–229] have been identified as contributors to the coloration of mAb solutions. As each mAb is different and the coloration may be caused by different factors, the major root cause of a strong coloration of a specific mAb solution needs to be evaluated on a case-

by-case basis. Although oxidation of tryptophan was the major source of the yellow color in one mAb solution,[138] the formation of AGEs resulted in a yellow color for a different mAb.[178]

Formulation buffer excipients could have a substantial impact on mAb coloration. For example, polysorbate, a commonly used excipient, has been shown to degrade over time when exposed to light and heat to generate free radicals or reactive oxygen species, leading to protein oxidation.[230,231] Oxidation of Trp could account for coloration of mAbs after exposure to heat and especially light.[138,178,221,232] The quality of polysorbate has a substantial impact on mAb photostability,[222] but its direct contribution to coloration at the time of release is minimal because of its low concentration. However, differences in polysorbate quality may become visible during forced degradation studies. Similarly, it has been demonstrated that mAb glycation can be caused by degradation of non-reducing sugar used in the formulation buffer,[172] which could contribute to coloration through the mechanism of advanced glycation end products,[178] especially under the relatively harsh conditions used for forced degradation studies.

Coloration of a mAb product itself may not be a concern, but it indicates chemical modifications of the products, which should be thoroughly investigated and the species giving rise to the coloration characterized, especially if the color is intense. In addition, product coloration variability can not only affect the assessment of the comparability of the pre- and post-change materials, but it can also affect clinical development, particularly the ability to blind clinical trials involving placebo controls.

1.14. Charge variants

From the perspectives of product quality and comparability, charge variants are important because they are the most commonly cited reason for heterogeneity.[3,4,233,234] A charge-based assay, either chromatographic or electrophoretic, is typically included in the panel of tests performed for drug substance release against defined release specifications. Charge-based assays are the most sensitive assays for detecting subtle differences caused by process changes.

In general, when analyzed by a charge-based method, the antibody charge variants are defined as either acidic or basic relative to the major species. Acidic species are variants with an apparent isoelectric point (pI) that is lower than that of the major species, while basic species are variants with an apparent pI that is higher than that of the major species. Acidic variants are typically the sum of unrelated mAb variants containing various degrees of sialylation, Asn deamidation and glycation.[3,4,233,234] Basic species are mainly formed due to uncyclized N-terminal Gln, C-terminal lysine and C-terminal amidation.[3,4,233,234] It is quite challenging to evaluate the comparability of pre- and post-change material from the perspective of charge variants because their overall differences with respect to structure, impact on product stability and biological function arise from the combined effects of the specific modifications discussed above and their relative levels. This is not to say that charge variants should be ignored in assessing comparability, but rather the assignment of product differences to a specific modification may be hard to ascertain. It is worth mentioning that different mAbs with pI differences greater than one pH unit did show differences in PK.[235] However, for charge variants of the same mAb, because the pI difference is less substantial, no difference in PK was observed when differently charged species were compared.[176]

When fractionated acidic, basic and the main species were tested in animal PK and pharmacodynamic (PD) studies, no differences were observed among the three groups of charge variants.[176] However, because charge variants are a feature common to all mAbs, they are required to be monitored by a release assay. If process changes result in differences in the formation of new product-related variants or impurities, the differences would likely be detected by a charge-based method.[32,225] When differences in charge profile are observed, thorough characterization is required to understand their chemical nature, and their impacts on safety and potency. If the potential impact on efficacy and safety cannot be inferred from the elucidation of the structure, additional *in vitro* and *in vivo* studies may be warranted.[32,225]

2. Product-related substance, impurities, process-related impurities and contaminants

Purified mAbs are inherently heterogeneous and should be regarded as a distribution of closely related molecular variants with slightly different molecular weights, charges, hydrophobicity and other properties such as N-glycosylation.[1-4] Per ICH Q6B,[7] variants of a mAb preparation can be defined as either product-related substances or product-related impurities. Product-related substances are "molecular variants of the desired product formed during manufacturer and/or storage which are active and have no deleterious effect on the safety and efficacy of the drug product." On the other hand, product-related impurities are defined as "molecular variants of the desired product (e.g., precursors, certain degradation products arising during manufacture and/or storage) which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety." This distinction is very relevant to the comparability exercise because the expectations for a tighter control of product-related impurities will be notably higher than for the variants. Likewise, the burden of proof necessary to justify the innocuous nature of a post-change difference that exceeds the acceptance criteria is higher for a product-related impurity than for a variant.

In addition to product-related substances and product-related impurities, ICH Q6B[7] also defines process-related impurities and contaminants. "Process-related impurities encompass those that are derived from the manufacturing process, i.e., cell substrates (e.g., host cell proteins (HCPs), host cell DNA), cell culture (e.g., inducers, antibiotics or media components) or down-stream processing." Contaminants are "any adventitiously introduced materials (e.g., chemical, biochemical, or microbial species) not intended to be part of the manufacturing process of the drug substance or drug product." As for product-related impurities, there are higher expectations for comparability justification of a post-change process-related impurity that exceeds the pre-change range of experience. The safety risks associated with process-related impurities and

contaminants call for particular attention to be paid to both when evaluating pre- and post-change materials for comparability.

The most common process-related impurities are HCPs, residual protein A and residual DNA that may cause concerns over immunogenicity and safety. In rare cases, trace amounts of proteases could degrade product and trace amounts of lipases could degrade polysorbates, leading to modification of the product. HCPs and residual protein A are measured using ELISA assays and residual DNA is measured by methods such as quantitative polymerase chain reaction (qPCR) for batch release and in-process monitoring. The information obtained from those types of assays is only a numerical value for each. A numerical value is sufficient for process changes that are unlikely to alter the impurity species, e.g., the same type of HCPs, residual DNA and residual protein A. However, substantial cell culture process changes may change the HCP and residual DNA populations or their relative abundance. While significant changes to protein A chromatography, including types of resins, buffers and operation conditions, may result in residual protein A impurities differing in size (e.g., hydrolyzed at different peptide bonds) or abundance. In-depth characterization may be necessary to demonstrate that not only the same amounts, but also the same types of impurity species are present in order to successfully demonstrate comparability.

3. Goal of comparability and phase-appropriate comparability

The goal of the comparability exercise is to demonstrate that the pre- and post-change products are comparable in terms of quality, safety and efficacy, as discussed in several guidance documents.[5,6,8,9] Specific attributes will be deemed comparable if they fit within a pre-defined acceptance window that are either agreed upon by regulators for commercial products or self-imposed but justified during earlier stage clinical development. Defining acceptance criteria are discussed later in this review.

The application of the comparability exercise is different depending on the stage of clinical development. Establishment of comparability allows nonclinical and clinical data generated using the pre-change product to be leveraged for the post-change product, enabling further clinical development and marketing application. In the case of a marketed product, the post-change product can be released to market without the necessity of additional clinical trials, thus avoiding the danger of negatively impacting the supply chain and affecting the availability of the drug to patients. In both cases, however, the objectives of comparability studies are the demonstration of the continuity and consistency of the quality attributes across the life cycle of the product.

Thus, the comparability exercise should focus on building a robust scientific argument supporting the linkage between process changes, and their potential effect on product quality, safety and efficacy by gathering and evaluating the totality of the data. The scope and extent of the comparability exercise will also depend on the nature of the required process changes. Comparability is established based on the evaluation of data from release testing, extended characterization of drug substance or drug product, in addition to process comparison, including appropriate process-controls and in-process data. Additionally, stability, variant isolation and characterization and forced degradation data could be included based on the phase of development, the extent of the process changes and the potential impact of process changes on product quality attributes. When changes in quality attributes exceed the expected range or cannot be rationalized in a scientifically sound manner, nonclinical and clinical studies are required to establish comparability to minimize the risk to patients in clinical and commercial settings.

Phase-appropriate comparability refers to the strategy adopted to ensure that the comparability study is designed to meet phase specific requirements (see Table 2), which vary in depth and scope for different phases of development.[8,9,236,237] Changes during the early phase (prior to Phase 2) are more acceptable due to limited product and process knowledge and clinical validation. Late-phase comparability (after Phase 2) is more comprehensive and stringent.[8] The most extensive and stringent comparability studies are those carried out after pivotal trials have been completed because the drug product manufactured using the post-change material is not intended to be used in clinical trials but for commercial supply.

Table 2. Scope of analytical comparability at different phases of development.

Phase of development	Scope of comparability	Acceptance Criteria
Nonclinical and Phase 1 clinical study	Release Characterization	Not necessary for pre-defined acceptance criteria
Between Phases 1, 2 and 3	Release Extended characterization (Including peak isolation and characterization if new peaks or the same peak with increased intensity are seen) In-process (assays and controls) Stability, if appropriate Forced degradation, if appropriate, selected conditions	Pre-defined acceptance criteria based on limited experience and limited statistical analysis
After pivotal study	Release Extended characterization (Including peak isolation and characterization if new peaks are seen) In-process (assays and controls) Stability Forced degradation, including more conditions	Pre-defined acceptance criteria based on statistical analysis

There is no need to carry out comparability studies prior to IND-enabling nonclinical studies, because the nonclinical animal study is the first opportunity to obtain *in vivo* data from the molecule. However, clinical trials are designed based on the nonclinical data; therefore, the materials used for nonclinical and clinical studies need to be comparable. As manufacturers gain more knowledge about the process and product during the clinical development phases (Phases 1, 2 and 3), comparability studies become more extensive and tighter acceptance criteria can be used. After pivotal trials, the comparability studies should be "as comprehensive and thorough as one conducted for an approved product."[8] and represents "the most challenging situation."[8]

4. Critical quality attributes

The definition of the critical quality attributes is the cornerstone to the quality-by-design approach outlined in ICHQ8 to Q10. Q8R(2)[238] instructs that "a CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality." Drug substance CQAs are assessed based on the quality attribute's effect on potency, safety and immunogenicity.[239]

The definition and assessment of mAb CQAs are not directly connected to drug substance or drug product process performance. However, manufacturing process conditions may influence the end product CQAs, and therefore must be considered. This is not only because extractable, leachable, cell culture additives, and other process-related residuals are potential CQAs, but also because process parameters have a substantial impact on product-related substance or product-related impurities. The relationship between process and drug substance CQAs needs to be thoroughly studied and determined during process development according to ICH guidance Q8(R2) and Q11.[238,240] CQA assessment is an iterative process performed a number of times across the clinical development life cycle as product knowledge increases. CQA assessment is aimed at establishing, in a systematic and rational manner, the impact of a particular variation on factors such as potency, PK/PD, immunogenicity and safety.

The assessment of CQA, followed by a process capability-driven risk assessment, is at the center of product development because it influences the control strategy, release and stability specifications, process characterization and validation and, last but not least, comparability studies. A risk assessment that considers the effects of the proposed process changes on all product CQAs must be conducted as part of the comparability protocol. Although comparability of non-CQAs may not have the same degree of stringency, they must be considered during comparability because they are valuable indicators of process robustness and consistency.

5. Risk-assessment

Risk assessment is a key component when planning process changes. During risk assessment, each CQA is considered in light of process capability and clinical consequences. Risk is defined as "the combination of the probability of occurrence of harm and the severity of that harm."[241] ICH Q9[241] describes risk assessment as an ongoing process, stating that "quality risk management is a systematic processes for the assessment, control, communication and review of risks to the quality of the drug (medicinal) product across the product lifecycle." Risk assessment is composed of risk identification, risk analysis and risk evaluation.[241] ICH Q9 states that "the evaluation of the risk to quality should be based on scientific knowledge and ultimately link to the protection of the patient".

Risk assessment is an integral part of a comparability study. The impact of the intended changes on product quality is evaluated based on knowledge about the product and process obtained from the development history and knowledge available from literature of the impact of similar changes on similar products. It is also very important to understand the relationship between raw materials, process parameters, and product quality with respect to structure-function, safety and efficacy. Examples of process changes and the associated potential risk are shown in Table 3. Naturally, each case is different and the risks need to be evaluated in light of the desired changes.

Table 3. Examples of proposed manufacturing changes and the associated risks.

Proposed changes	Potential risks to CQAs
Manufacturing site change	High risk
Drug substance scale change	Medium risk
Facility fit change	Medium risk, depending on the nature of change
Cell line change	High risk
Medium and feed change	High risk
Fermentation set point change	Medium to low risk, may have been covered during process characterization
Chromatography matrix change	High risk on clearance of residuals, adventitious agents, product-related substances/impurities and process-related impurities
Chromatography operation parameter change	Medium risk, may have been studied during process characterization
Raw material changes	Medium risk, potentially impacting extractable and leachable
Drug substance storage container and temperature	Medium risk, extractable, leachable, stability
Formulation change-new excipients	High risk, stability
Formulation change-same excipients at different concentrations	Low risk
Drug product storage temperature	Low risk, supported by development data
Drug product packing	Low risk, supported by development data
Drug product presentation	Medium to high risk, depending if raw material or device are changed

Risk assessment helps define the extent of comparability studies, driving the selection of lots, analytical methods, and what studies (e.g., extended characterization, forced degradation) are required. For example, the extensiveness of comparability for a change affecting a non-CQA is expected to be much less than a change affecting a CQA. ICH Q5E[8] states that "a careful consideration of potential effects of the planned change on steps downstream and quality parameters related to these steps is extremely important." The FDA guidance on comparability protocols also suggests including a risk assessment of the proposed change in the submission because it identifies the potential effects of the proposed changes on product quality.[242]

6. Scope of comparability

The core data used for the comparability study come from routine batch release testing, extended characterization and process comparison in terms of process controls and in-process results. Depending on the phase of development, the nature of changes, and the outcome of the risk assessment, stability and forced degradation data may also be needed to establish comparability. In cases where comparability cannot be established based on quality data, nonclinical and clinical studies are required.[8,237]

In addition to the phase of development, other factors, including the nature of the changes (location and extent), the potential impact of the change on product quality, process capability, the suitability of the analytical methods, MOA of the mAb and the relationship between product quality, safety and efficacy, are also critical factors to consider when designing a comparability study.[5,6,8,242]

Process parameters that are within the normal operating ranges or are within the design space pre-approved by regulators are out of scope for comparability assessment. The foregoing point notwithstanding, it is the responsibility of manufacturers to inform or seek approval from regulators prior to the implementation of the process change at all stages of development, regardless of the nature of the process change. For changes to process parameters that fall within ranges covered during process characterization and linking studies, the scope of the comparability study can be reduced based on the output of the risk assessment.

6.1. Routine batch release

The analytical methods used for release testing are chosen to confirm the product quality,[7,8] and should be capable of directly or indirectly monitoring all CQAs. Therefore, the evaluation of pre- and post-change batch release data is essential to establishing comparability. A typical set of release assays for mAbs are listed in Table 4. These assays measure the general properties, safety, purity, potency, identity, charge and glycosylation of the product. Specifications for each monitored product attribute are set while taking into account process capabilities, product stability, preclinical and clinical data, and analytical method capabilities.[7] Meeting the release specifications is a basic requirement for demonstrating product consistency with respect to product quality, safety and efficacy.

Bioassay, typically included in the release assay panel, is specifically mentioned in several ICH, FDA and EMA guidance documents,[5–8,243] because it serves a variety of purposes. First, the bioassay measures the activity of the product, which is an important quality attribute. Second, the bioassay ensures the integrity of the mAb with respect to its higher order structure, especially when the direct assessment of the structure is challenging. Third, the bioassay serves as "a link to clinical activity."[8] The bioassay can also provide other useful information, for example "when a drug substance has more than one form and a manufacturing change shifts the distribution of forms, determination of the bioactivity of the various forms may be of value in assessing the impact of the change."[5] Of particular interest is the guidance from ICH Q5E,[7] which states that "when changes are made to a product with multiple biological activities, manufacturers should consider performing a set of relevant functional assays designed to evaluate the range of activities." This is especially true for a mAb that targets a cell surface antigen and requires the engagement of a Fcγ receptor or complement proteins as part of the MOA.[98] In such a case, ADCC or CDC activity may need to be evaluated.

Comparison of the nature and levels of impurities in lots manufactured pre- and post-change are also discussed in guidance documents.[7,8,242] For all mAbs, product-related impurities include aggregates, which are detected by SEC-HPLC. Process-related impurities include HCPs, residual DNA, residual protein A, and other chemicals used in cell culture and purification. Impurities may not only impact efficacy, but, even more importantly, they may pose a safety risk if elevated levels or new impurities are detected in the post-change lots.

6.2. Extended characterization

While the release tests are designed to confirm the basic product quality, they do not provide a detailed characterization of the biophysical, biochemical and functional properties of the molecule.[7,8] Therefore, additional extended characterization assays (Table 5) are needed for an in-depth understanding of the product.[7,8,242] Characterization assays are selected to detect the potential impact of process

Table 4. A typical list of batch release assays for mAb drug substance.

Attributes	Methods
Safety	Bioburden
Safety	Endotoxin
General	Appearance (color and clarity)
General	pH
General	Concentration
Identity	Peptide mapping (LC-UV)
Purity	SDS-PAGE/CE-SDS (non-Reducing and reducing)
Purity	SEC-HPLC
Potency	Antigen binding
Potency	Cell-based assay
Potency	Effector functions*
Charge/identity	IEX-HPLC/IEF/cIEF/CZE
Glycosylation	N-glycan profiling by NP-HPLC of labeled glycans
Impurities	HCPs
Impurities	Host cell DNA
Impurities	Residual protein A

*If involved in the mechanism of action.

Table 5. A typical list of extended characterization assays and the information they provide.

Extended Characterization Assays	Attributes to detect
LC-MS or CE-MS analysis of intact mAb	Primary structure and PTMs such as glycosylation, glycation (may need deglycosylation)
LC-MS or CE-MS analysis of reduced mAb	Primary sequence and PTMs such as glycosylation, glycation
LC-MS or CE-MS analysis of IdeS digested antibody with/without reduction	Primary sequence and PTMs such as glycosylation, glycation
LC-MS or CE-MS analysis of peptide mapping	Primary sequence, PTMs such as oxidation, deamidation, glycation
Free cysteine	Amount of free cysteine
Ion mobility mass spectrometry	Aggregates, monomer and fragments
Hydrophilic interaction chromatography with fluorescence and MS detection	Glycan profiling, detection and quantitation of immunogenic glycans (e.g., NGNA and α-1,3 Galactose)
Disulfide bond confirmation	Confirmation of the correct disulfide bond linkage
Circular Dichroism	Secondary and tertiary structures, conformation
Analytical ultracentrifugation	Aggregates, monomer and aggregates; conformation
Differential scanning calorimetry	Tertiary and quaternary structures indicated by thermal properties (transition temperatures)
Asymmetrical flow field-flow fractionation	Aggregates, monomer, and fragments
Micro-flow imaging or HIAC	Particles
Surface-plasma resonance	Antigen, Fc receptor or FcRn binding affinity
Flow cytometry	Fc receptor and C1q binding
Plate-based formats (e.g., ELISA)	Fc receptor and C1q binding

changes on product quality, mAb structure, biological activity, and impurity profile.

Extended characterization assays are either orthogonal to release methods or state-of-the-art technology with the capability to detect subtle differences. In contrast to release methods that need to be appropriately qualified or validated according to the phase of development, extended characterization methods need not be validated, but should be scientifically sound and appropriately controlled, with the underlying science qualifying these methods for detection of quality attributes that could be challenging by release methods. For example, analytical ultracentrifugation (AUC) is orthogonal to size-exclusion chromatography for determination of the percentage of aggregates, monomer, and fragments in solution. Additionally, AUC determines the hydrodynamic size distribution of the molecules, which is a reflection of the molecule's conformation. Molecular weight measurements using mass spectrometry can provide not only the accurate molecular weight, but also the relative percentage of PTMs. Circular dichroism is a method that measures the secondary and tertiary structures of the mAb molecule, information that is not typically obtained by release assays.

It is worthwhile to mention, if new species are detected in the post-change products, isolation and in-depth characterization of the new species is warranted to understand any specific modifications. Depending on the identified modifications, further *in vitro* or *in vivo* studies may be required to evaluate the impact on safety and efficacy.

Additional analytical approaches, such as sequence variant analysis by mass spectrometry and protein conformational assessment by hydrogen-deuterium exchange mass spectrometry, could be evaluated and implemented to provide in-depth characterization to further ensure comparability when major process changes were introduced, such as a cell line change.

6.3. Process performance comparison

FDA guidance[5] recognizes that "because of the limited ability to characterize the identity and structure and measure the activity of the clinically-active components(s), a biological product was often defined by its manufacturing process." It also states that "improvements in production methods, process and control test methods, and test methods for product characterization have led to the evolution of the regulation of biological products."[5] ICH Q11[240] discusses the relationship between material attributes and process parameters and drug substance CQAs. In addition to the critical roles of in-process controls and in-process testing, modern analytical techniques enable thorough characterization of biological products.[5] The combination of a well-controlled process and thorough characterization further ensures production of consistently safe and efficacious product.[7,9,236,242,243]

Process controls are designed to ensure consistent process performance. "Critical control points in the manufacturing process that affects product characteristics,"[8] (ICH Q5E) should be evaluated, defined and monitored. ICH Q5E[8] also states that "a careful consideration of potential effects of the planned change on steps downstream and quality parameters related to these steps is extremely important (e.g., for acceptance criteria, in-process specifications, in-process tests, in-process hold times, operating limits, and validation/evaluation, if appropriate)." For the post-change process, ICH Q5E[8] states that "in-process controls for the post-change process should be confirmed, modified, or created, as appropriate, to maintain the quality of the product."

In-process testing ensures that the process performs as planned, and data from in-process testing provides additional evidence of comparable product.[5,8,244] Products should be evaluated at the most appropriate steps to enable detection of a change[8] and at the steps most likely affected by the process change.[5] ICH Q5E[8] states that, with regard to comparability, the manufacturer should evaluate "results from analysis of relevant samples from their appropriate stages of the manufacturing process (e.g. intermediate, drug substance and drug product)." It further states that: "to support the comparison, it is often useful to demonstrate, for example, that specific intermediates are comparable or that the modified process has the capability to provide appropriate levels of removal for process- and product-related impurities, including those newly introduced by the process change".

Step-by-step in-process comparison provides strong assurance that drug substances manufactured by the pre-change and post-

change processes are comparable. However, such a comparison may not be meaningful for some down-stream changes. For example, if a new type of column is introduced to increase aggregate clearance, it may simultaneously decrease HCP clearance. In this case, it is critical to demonstrate that subsequent purification steps have the ability to remove the potentially higher HCP level in order to produce in-process samples or drug substance with equal or lower level of HCPs than those of the original process. As we discussed in the section on product-related substance, impurities, process-related impurities and contaminants, significant changes to the process may necessitate an in-depth characterization of the specific impurity species.

6.4. Stability

Stability studies are used to demonstrate that the post-change material has a stability profile comparable to that of the pre-change material. In addition, they have the potential to detect differences that cannot be detected by release and extended characterization assays[8] at the time of manufacturing, such as changes in protein structure, or purity /impurities (ICH Q5E).[8] FDA guidance on comparability protocol[242] states that "stability studies (e.g., real condition, forced degradation) may also be appropriate and should provide a direct comparison of products manufactured before and after the change to ensure that the product will maintain quality throughout its shelf life after implementation of the proposed change (s)". ICH Q5E[8] emphasizes "the need for stability data, including those generated from accelerated or stressed conditions, to provide insight into potential product differences in the degradation pathways of the product and, hence, potential differences in product-related substances and product-related impurities." ICH Q5E further suggests initiating real time/real temperature stability studies based on the rationale that changes in protein structure, or purity /impurities may affect stability, and that stability studies might detect subtle differences that cannot be detected by other methods. It also mentions that "accelerated and stress stability studies are often useful tools to establish degradation profiles and provide a further direct comparison of pre-change and post-change product."

Data trending is sufficient for the purpose of expiry determination. However, to support comparability, comparison of chromatograms, gel images and other qualitative data may be necessary to demonstrate the same degradation pathways of the pre- and post-change materials.

6.5. Forced degradation

Forced degradation studies, typically carried out under relatively harsh and highly relevant conditions are recommended to support comparability.[8,242] The variety of conditions used for forced degradation increases the likelihood of detecting differences by probing the degradation pathways most relevant to the mAb of interest. Forced degradation studies allow a side-by-side comparison of pre- and post-change lots within a short time period by generating relatively high levels of degradation for better comparison of degradation products and kinetics.

The need to include forced degradation studies to support comparability is highly dependent on the stage of the project and also on the process steps where the changes were introduced. Forced degradation studies may not be needed for comparability studies carried out at early stages. If the changes are simple and the impact on quality attributes can be predicted, forced degradation studies may not be needed or may be performed using only select conditions that can be justified based on the predicted impact.

A pre-screening study is normally required to identify the relevant and optimal conditions, as well as appropriate analytical methods to assess product degradants. The optimized conditions should result in reasonable levels of degradation by the most sensitive methods in order to provide degradation kinetics. The degree of degradation achieved should depend on the sensitivity of the molecule to stress and the capability of the selected methods to detect the expected degradation products. A good balance should be struck between achieving sufficient degradation levels for differentiation between pre- and post- change products, avoiding too excessive degradation, which may be due to secondary degradation effects.

With the exception of light-induced stress, no guidance documents, for good reason, explicitly states the nature of the stress and the conditions to be used for forced degradation studies. Examples of forced degradation conditions and the expected impact on mAb are presented in Table 6. Similar information has been reported in the literature.[245]

7. Comparability protocols

A comparability protocol describes in detail the assays, studies, and acceptance criteria that will be used to assess the effect of one or more CMC changes on product quality.[242] Though practices vary from company to company with regard to the need of a comparability protocol for pre-commercialization projects, a comparability protocol provides an organized, systematic way to carry out the comparability studies. The basic content of such a protocol is described in Table 7.

8. Lot selection

Although, it is common to include three pre-change and three post-change lots for a typical comparability study, the number of lots is only vaguely described in guidance documents. An FDA document[6] states that "comparisons should test a number of separate product lots in parallel in order

Table 6. Various forced degradation conditions and their effects on mAbs.

Forced degradation conditions	Quality attributes to evaluate
Thermal	Aggregations and chemical modifications such as oxidation, deamidation
Low pH	Aggregation and fragmentation
High pH	Aggregation, deamidation, degradation of disulfide bonds
Agitation	Aggregation
Freeze/thaw	Aggregation
Oxidation	Susceptible sites of oxidation, which may be altered if structure changes introduced
Deamidation	Susceptible sites of deamidation, which may be altered if structure changes introduced
Glycation	Susceptible sites of glycation, which may be altered if structure changes introduced
Photo	Tryptophan oxidation

Table 7. Contents of a typical comparability protocol.

Sections	Contents
Process history and comparison	Brief process history
	Rational for process change
	Comparison of pre- and post- change process
Risk assessment	Leverage on development knowledge and scientific literature to predict which quality attributes are likely to be impacted and the potential impact on safety and efficacy
	Leverage knowledge of CQA for this risk assessment
Comparability strategy	Release
	Extended characterization
	In-process
	Stability, if needed
	Forced degradation, if needed
	Non clinical and clinical, if needed
	Provide justifications for the selected tests and studies
	Number of lots
Lot selection	Lot genealogy
	Representative lots of the pre- and post-change lots
Methods and studies	List of methods, studies and justification
Acceptance criteria	Quantitative and qualitative

to demonstrate the reproducibility of the new manufacturing scheme." ICH Q5E[8] states that "for approved products, an appropriate number of post-change batches should be analyzed to demonstrate consistent performance of the process." It also mentions the use of "historical data that provide insight into potential "drift" of quality attributes with respect to safety and efficacy." ICH guidance [240,244] suggests testing "relevant drug substance batches". The use of more than one lot provides the advantage of demonstrating process robustness; however, it may not be feasible or necessary, especially for projects in the development phase.

When deciding on the number of lots to include, several factors are worth considering. These include phase of development, the type of change, and understanding of the process and product. Prior to process validation, the number of lots manufactured is most likely defined by the clinical demand. It may not be feasible to include more lots in the comparability study. In addition, for minor changes, one lot is acceptable.[236] However, after a pivotal trial, a minimum of three pre-change and three post change lots may need to be included in the side-by-side analytical comparability evaluation (extended characterization and forced degradation). Release data from historical lots should be included for a statistical evaluation of product quality attributes.

Regardless of the number of lots included in the comparability studies, they need to be representative of the pre- and post-change manufacturing experience and ideally have received sufficient clinical exposure.

9. Acceptance criteria

Setting pre-defined and meaningful comparability acceptance criteria is recommended in the guidance documents.[6,8] The major advantage of pre-defining acceptance criteria is to ensure an objective evaluation of the data.[8] The FDA guidance[242] on comparability protocol recommends "relevant and clearly defined acceptance criteria", including those of impurity profiles, stability studies, and any other studies. It is highly beneficial to have pre-determined acceptance criteria for comparability studies during early development phases, with the understanding that the acceptance criteria cannot be as well defined as late-stage projects because of limited experience during the early development phase.[246] Examples of attributes and associated acceptance criteria that might be used are shown in Table 8.

When setting up criteria, the major factors to consider are nonclinical and clinical experience gathered during the early phases of development, the relationship between quality attributes and safety and efficacy, process knowledge, and an understanding of the analytical methods used for lot release and extended characterization. Additionally, the control strategy, risks associated with proposed change, and the intended use of the product should be considered.[242] When a sufficient number of pre- change lots are available, appropriate statistical methods should be used to set acceptance criteria.[242]

One of the common questions with regard to comparability assessment is, "how comparable is comparable?" ICH Q5E[8] requires the pre- and post-change products to be "highly similar". According to Q5E, "the demonstration of comparability" does not require identical quality attributes, but that "the existing knowledge is sufficiently predictive to ensure that any differences in quality attributes have no adverse impact upon safety or efficacy of the drug product". Therefore, as discussed in earlier sections, general knowledge of mAbs, specific knowledge gained during the development of the specific mAb of interest (e.g., characterization, process development, stability, forced degradation, nonclinical and clinical), MOA, and CQAs provide the basis to establish meaningful acceptance criteria and thus facilitate product comparability assessment.

It is worthwhile to mention that ICH Q5E[8] states that "results within the established acceptance criteria, but outside historical manufacturing control trends, might suggest product differences that warrant additional study or analysis". On the other hand, FDA guidance[242] on comparability protocols states that "the acceptance criteria for the change can allow for differences in product attributes if you provide justification based on your assessment of the effect(s) of the change on safety and effectiveness. If you anticipate such differences, they should be prospectively described."

9.1. Routine batch release

It is a prerequisite to meet release specifications in order to release a lot; however, meeting release specification in itself is not sufficient as the sole acceptance criteria for comparability because specifications are designed to confirm to the routine quality attributes of the products.[7,8] Acceptance criteria for comparability should be set based on evaluation of the release data with numerical values using an appropriate statistical approach, when a sufficient number of lots have been manufactured.[242] In addition, acceptance criteria should be set based on qualitative evaluation of the chromatographic or electrophoretic profiles of the corresponding methods.

Quantitative acceptance criteria are set for those assays that report numerical values. Data from all historical lots may be considered in addition to those from the lots included in the comparability study, especially those lots used in clinical studies. It is also suggested that an appropriate statistical approach

for data analysis be used, especially for late-stage comparability studies when a sufficient number of lots have been manufactured.[242] For early-stage comparability, acceptance criteria should be set based on non-clinical and clinical experience, while taking into account the limited experience with the process and the analytical methods. Scientific understanding of mAbs, including knowledge and experience with the various PTMs, structure, stability and MOAs, plays a critical role in setting acceptance criteria. For example, oligosaccharide differences in the Fc region for a mAb targeting a soluble target are not as critical as for an antibody targeting a membrane-bound target,[98] and thus there may exist reasonably wide acceptance criteria for this particular attribute.

Qualitative acceptance criteria are set based on comparison of features such as peak profiles, and banding patterns. For example, SDS-PAGE has been commonly used to determine product purity. This method provides the percentage of the main bands of intact molecule under non-reducing conditions and heavy chain and light chain under reducing conditions, but additional bands should also be compared. The presence of the same number of the additional bands at the same migration times should be included as an acceptance criterion. The presence of a new band or bands with different migration times may indicate the presence of new impurities, or new degradation products.

ICH Q6B[7] states that "the manufacturer should define the pattern of heterogeneity of the desired product and demonstrate consistency with that of the lots used in preclinical and clinical studies." If the pattern is consistent, evaluation of each form is not necessary.[7] However, "when process changes and degradation products result in heterogeneity patterns which differ from those observed in the material used during preclinical and clinical development, the significance of these alterations should be evaluated."[7] Such statements are highly applicable to mAbs because their inherent heterogeneity potentially contains both product-related substances and product-related impurities.

Special attention should be given to the comparison of the impurity profiles both quantitatively and qualitatively. FDA guidance[242] on comparability protocol recommends "to determine any qualitative and quantitative changes to the impurity profile of the drug substance, product, intermediate, in-process material, or other material manufactured using the new process." and that "you should demonstrate an understanding of the origin and risk of any new or increased level of impurities or contaminants." Increased levels of the same impurities in post-change product poses a significant safety risk because the levels are not qualified by the previous phases of development. Furthermore, the appearance of new impurities poses an even higher risk.[8] It is a general expectation that the level of impuri-

Table 8. Proposed acceptance criteria for mAb comparability assessment.

Category of testing	Specific assays	Acceptance criteria
Routine batch release	Peptide mapping	• Meeting release specification •Comparable peak profiles based on retention times and relative intensity •No new or missing peaks in the post-change lots
	SDS-PAGE/CE-SDS	•Meeting release specification •Percentage of main band/peak within the acceptance criteria based on statistical analysis •Same banding/peak pattern •No new species
	SEC-HPLC	•Meeting release specification •Percentage of main peak within the acceptance criteria based on statistical analysis •Same retention times of the aggregate, monomer and fragment peaks
	Charge (CEX, cIEF)	•Meeting release specification •Percentage of major peaks within the acceptance criteria based on statistical analysis •No new peaks in the post-change lots
	Oligosaccharides	•Meeting release specification •Percentage of major peaks within the acceptance criteria based on statistical analysis •No new peaks in the post-change lots
	Binding affinity	•Meeting release specification •Binding affinity within the acceptance criteria based on statistical analysis
	Cell based assay	•Meeting release specification · Potency within the acceptance criteria based on statistical analysis
Extended characterization	Molecular weight analysis by LC-MS	•Mass error within the instrument accuracy •The same species
	Peptide mapping with LC-MS detection	•Confirmation of the primary sequence •Percentages of post-translational modifications within the acceptance criteria
	Disulfide bonding pattern	•Confirmation of the correct disulfide bond linkage
	Free thiol	•Level of free cysteine within the acceptance criteria based on statistical analysis
	CD	•No substantial difference in the spectra and conformational fractions, if calculated
	AUC	•Percentage of main peak within the acceptance criteria based on statistical analysis •Aggregates, monomer, and fragments with comparable sedimentation velocity
Process comparison	Process controls	•Equal or better process control
	Product quality	•Equal or better impurities clearance •Equal or better product intermediate stability •Comparable product-related substance
Stability	Real time and accelerated	•Comparable or slower degradation rates •Same degradation pathways
Forced degradation	Various conditions	•Comparable degradation kinetics •Same degradation pathways

ties in the product manufactured using the post-change process should be within the statistical range of clinical experience.[236] The presence of the same types of impurities can be reasonably assumed based on the same peak profiles, e.g., aggregates by size-exclusion chromatography (SEC). For other impurities, confirming the presence of the same types of impurities is challenging and may not even be necessary. For example, when measuring HCP concentration, only a numerical value is reported. It is not required, nor is it a common practice, to compare each individual HCP species for comparability, unless the process change is motivated by the need to remove a particular HCP. For most incremental changes not involving drastic changes in the chemistry of the chromatography step or the buffers used, the same type of HCPs can be reasonably assumed based on comparable processes. The appearance of a new impurity is highly undesirable. ICH Q5E[8] states that "where the change results in the appearance of new impurities, the new impurities should be identified and characterized when possible. Depending on the impurity type and amount, it might be appropriate to conduct nonclinical or clinical studies to confirm that there is no adverse impact on safety or efficacy of the drug product." ICH Q5E also states that "new impurities could warrant toxicological studies for qualification."

Results from routine release tests such as appearance (color, clarity, visible particulates) should not be ignored in assessing the comparability of pre- and post-change materials because they can be early indicators of quality attributes that have gone awry.

9.2. Extended characterization

Similar to release assays, acceptance criteria for extended characterization assays are set based on both quantitative and qualitative evaluation. For quantitative evaluation, statistical analysis of the characterization data may not be applicable because of limited characterization data from a limited number of lots. In this case, setting acceptance criteria may be mainly based on understanding of the quality attributes that the assays measure, the scientific underpinning of the specific assays and their demonstrated variability.

For example, liquid chromatography- mass spectrometry (LC-MS) is used to measure the molecular weight of the mAb and its variants. Based on the generally accepted mass accuracy of the method, variation in the molecular weight exceeding a reasonable range (e.g., 2 Da) is not acceptable because it indicates amino acid sequence differences.[247] However, mass accuracy is highly dependent on the type of mass spectrometer,[248] and thus needs to be documented and verified for the specific instrument that is used to generate comparability data.

Setting quantitative criteria for circular dichroism poses other kinds of challenges. Although the data can be deconvoluted to estimate the percentage of the structural features or conformational fractions of a mAb in solution, these numbers are far from accurate as they are derived from algorithms generated for a reference set of X-ray crystallography data. With the understanding of this method, setting descriptive acceptance criteria may be sufficient because meaningful differences can be recognized by experienced analysts.

In addition to setting quantitative criteria, it is also necessary to set qualitative acceptance criteria to demonstrate the presence of the same molecular species in the pre- and post-change lots. AUC is a commonly used extended characterization assay. In addition to determining the percentage of aggregates, monomer and fragments, it measures the sedimentation velocity of various species. The same sedimentation velocity values of the respective species in the pre-change and post-change lots indicate the presence of the same types of aggregates, monomers and fragments.

The development of new methods will continue to expand the number of extended characterization methods that can be used and further ensure even more in-depth characterization and subsequent comparability assessment. Analytical approaches that have the potential to revolutionize comparability assessment include the use of multiple attribute methods,[249-252] 2D-LC-MS,[253,254] hydrogen-deuterium exchange-MS [255]and nuclear magnetic resonance.[256] Such new analytical approaches will require new kinds of acceptance criteria, such as percentage overlap, to be developed.

9.3. Process comparison

Acceptance criteria are set for both process parameters and in-process product quality attributes. ICH Q5E[8] states that "when changes are made to a process, the manufacturer should demonstrate that the associated process controls, including any new ones, provide assurance that the modified process will also be capable of providing comparable product." It goes on to instruct that "the manufacturer should confirm that the process controls in the modified process provide at least similar or more effective control of the product quality, compared to those of the original process," especially at "critical control points" that have the ability to affect product characteristics.

ICH Q5E [8]states that "to support the comparison it is often useful to demonstrate, for example, that specific intermediates are comparable or that the modified process has the capability to provide appropriate levels of removal for process- and product-related impurities, including those newly introduced by the process change". For a typical mAb process, the impact of process change on product variants (e.g., charge profiles), product-related impurity clearance (e.g., aggregates and fragments) and process-related impurity clearance (e.g., HCP, DNA, Protein A) should be compared.

It is expected that, at a minimum, materials produced from the post-change process should have comparable or better quality compared to the materials produced from the pre-change process. It is straightforward to compare quantitatively and qualitatively product-related variants and product-related impurities because quantitative and qualitative information can be readily obtained from analysis of in-process samples using techniques such as SEC for aggregates and fragments, and charge-based methods for charge profile. However, it is challenging to qualitatively compare process-related impurities such as HCPs, residual DNAs and residual protein A because the routine assays can only provide numerical values. For process changes, such as scale-up, comparison of just the numerical values is sufficient because qualitative changes are not expected. However, for other changes such as significant cell culture changes or down-stream changes that can potentially change HCP species, residual DNA species, or the residual protein A degradation products, qualitative comparison may be

necessary to demonstrate the same process-related impurity species in the post-change materials.

Where a step-by-step comparison is not feasible, such as in the case of significant down-stream changes, evaluation based on the totality of in-process clearance of product-related impurities and process-related impurities could be very helpful. Demonstration of the same level or a lower level of product-related and process-related impurities in in-process samples prior to the final ultrafiltration/diafiltration provides further assurance of comparable products with regard to impurities.

9.4. Stability

Passing stability specifications is a prerequisite but insufficient to demonstrate comparability. Acceptance criteria for stability comparisons are set based on both quantitative and qualitative criteria. Quantitative criteria are set based on comparable degradation kinetics as demonstrated by comparable slopes of degradation over time. Qualitative acceptance criteria are set based on the same degradation pathways as demonstrated by the appearance of the same chromatographic peaks or electrophoretic bands, which may be readily detected in the accelerated stability samples, but not as easily in the real time stability samples because of relatively low or undetectable levels of degradation. In cases where unique peaks or bands are only detected in pre-change or post-change lots, further characterization is needed to thoroughly understand the degradation products, identify the root cause of the differences and evaluate their impact on product quality, safety and efficacy.

9.5. Forced degradation

Similar to stability acceptance criteria, quantitative acceptance criteria for forced degradation are set based on the comparison of degradation kinetics (e.g., loss of monomer, loss of activity). Qualitative acceptance criteria are set based on products having the same degradation pathways with the assumption that peaks with the same retention times or bands with the same migration times indicate the same degradation products.

In cases where unique degradation products are detected only in pre-change or post-change lots, the unique peaks need to be isolated and characterized to determine the nature of the degradation products. Identification of the unique degradation product will be used to evaluate potential differences in the product and their impact on safety and efficacy.

10. Comparability outcomes

ICH Q5E[8] states that "generally, quality data on the pre- and post-change product are generated and comparison is performed that integrates and evaluates all data collected, e.g., routine batch analyses, in-process control, process validation/evaluation data, characterisation and stability, if appropriate."

Ideally, comparability is established based on quality data evaluation as demonstrated by meeting the predefined acceptance criteria. In such cases, no additional studies are needed. However, the same outcome may be reached when attributes in pre- and post-change are outside of the acceptance criteria, if the differences can be justified as having no adverse impact on safety and efficacy based on the manufacturer's knowledge of the molecule and scientific knowledge found in literature. A rationale will need to be provided when comparability is being claimed in the face of very tight acceptance criteria that were not met. Explanation as to why the predefined acceptance criteria were unnecessarily tight would have to be provided and justified.

In other cases, predefined acceptance criteria based on historical experience, may not be met because a process change results in an improved product quality. ICH Q5E[8] affirms this as positive change when it states that "improvement of product quality is always desirable and encouraged". If the product quality improvement only positively impacts safety (e.g., decreasing the levels of HCPs, aggregates), the pre- and post-change products may be considered comparable. However, if the product improvement results in significant benefit in efficacy, the pre- and post-change products may not be deemed comparable. In such cases, the manufacturer is advised to consult the appropriate regional regulatory authority for possible subsequent actions.

If comparability could not be established based on quality data evaluation alone, further nonclinical and clinical studies would be required.[5,6,8,237] Although not the focus of this review, some of these situations are summarized in Table 9.

11. Data presentation

Upon completion of a comparability study, a comparability report is generated and ultimately used for regulatory submission to obtain approval of the changed process. The key to summarizing a comparability study is to present a complete scientifically sound story that accurately reflects the data. Comparability reports may be organized according to the comparability protocol with the addition of results and

Table 9. Requirement for nonclinical and clinical studies.

Situations	Possible causes
Differences are expected, although not detected	Analytical methods not capable to detect the expected difference
Differences identified and impact on safety and efficacy cannot be ruled out	Lack of understanding of the difference or lack of strong structure-function relationship if the chemical nature of the differences identified
Differences identified and potentially impact on the safety and efficacy	Existing knowledge indicating the difference potentially cause safety and efficacy concern
Appearance of new impurities, adventitious agents	Depending on the type and amount of impurity, no adverse impact on safety and efficacy cannot be ruled out
Immunogenicity is a major concern	For example, elevated amount of non-human moieties, new impurities or impurities level higher than experienced in clinical studies

summary. If there are differences, the report should primarily focus on the differences and justifications as to whether or not the differences will negatively impact product quality, and thus adversely impact product safety and efficacy.

Data generated by orthogonal methods should be discussed, taking into account the similarity and differences of the specific methods. For example, there are at least three methods from release and extended characterization that have the capability to detect aggregates. These are SEC, SDS-PAGE (or CE-SDS) and AUC. However, differences in aggregates as measured by these three methods are expected owing to the fact that each method is based on different biophysical and biochemical principles. Thus, results should be considered in light of the limitations and specificities of each method.

Presenting raw data such as chromatograms and gel images is very helpful for direct visual comparison. It is worthwhile to mention that for direct comparison, lots included in comparability studies should be analyzed side-by-side on the same gel or within the same HPLC sequence to eliminate the impact of method variability on the results. Chromatograms and gel images of the same size and scale of pre-change and post-change lots should be a basic requirement. At a minimum, all labels should be legible. For trending plots, different colors and symbols should be used for the corresponding pre- and post-change lots consistently throughout the document to facilitate review.

12. Comparability vs. biosimilarity

The comparability principle outlined in ICH Q5E is applicable to "products where manufacturing process changes are made by a single manufacturer, including those made by a contract manufacturer, who can directly compare results from the analysis of pre-change and post-change product." In contrast, a biosimilar product is made by a different company other than the originator. Establishing biosimilarity requires more extensive data generation than establishing comparability.[257]

The US FDA has issued guidance for the industry that provides very useful information on scientific and quality considerations for demonstrating biosimilarities.[258,259] In these guidance documents, the FDA acknowledges the difficulty of demonstrating biosimilarity and clarifies the limitations of comparisons during this exercise with a comparability exercise after a manufacturing change. Part of this complexity lies in the fact that the innovator's product information remains proprietary and is not available to the biosimilar manufacturer. Therefore, the biosimilar manufacturer begins with only the final product and must work to develop a process to produce the biosimilar product. In contrast, a manufacturer conducting a comparability exercise following a manufacturing change in one of its existing products will begin with extensive knowledge and history of the product.[260]

13. Conclusion

Changes are common throughout the lifecycles of recombinant monoclonal therapeutics. Comparability studies are carried out for each manufacturing process change to demonstrate that the post-change lots are comparable to the pre-change lots as defined by having no adverse impact on safety and efficacy. The scope and extent of the comparability exercise depend, to a very large extent, on the stage of development and the nature of the changes. The comparability exercise is intended as a systematic review of the potential impact of process changes on safety and efficacy by leveraging CQAs of the product and by conducting risk assessment. Defining the scope of the comparability study, setting suitable acceptance criteria, executing the studies and summarizing the results are all critical aspects of a comparability study. Carefully crafted and executed comparability studies are necessary to ensure continuous manufacturing of safe and efficacious products.

Abbreviations/acronyms

ADA	Anti-drug antibodies
ADCC	Antibody-dependent cell-mediated cytotoxicity
AGE	Advanced glycation end products
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
C1q	The first component of complement
CD	Circular dichroism
CDC	Complement-dependent cytotoxicity
CDR	Complementarity-determining regions
CH2	Constant domain 2
CHO	Chinese hamster ovary
cIEF	Capillary isoelectric focusing
CQA	critical quality attributes
Cys	Cysteine
CZE	Capillary zone electrophoresis
DSC	Differential scanning calorimetry
EMA	European Medicines Agency
Fab	Antigen-binding fragments
Fc	Fragment crystallizable region
FcγR	Fc gamma receptor
FcRn	Neonatal Fc receptor
FDA	US Food and Drug Administration
GlcNAc	N-acetylglucosamine
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
HCP	Host cell protein
His	Histidine
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IEF	Isoelectric focusing
IEX	Ion exchange chromatography
IgG	Immunoglobulin
IND	Investigational new drug
IsoAsp	Isoaspartate
LC-MS	Liquid chromatography-mass spectrometry
Lys	Lysine
mAb	Recombinant monoclonal antibody
Met	Methionine
MOA	Mechanism of action
NANA	N-acetylneuraminic acid
NGNA	N-Glycolylneuraminic acid

PD	pharmacodynamics
pI	Isoelectric point
PK	Pharmacokinetics
PTM	posttranslational modifications
PyroGlu	Pyroglutamate
SEC-HPLC	Size exclusion chromatography-high performance liquid chromatography
Thr	Threonine
Trp	Tryptophan

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