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Review paper

# Basic regulatory science behind drug substance and drug product specifications of monoclonal antibodies and other protein therapeutics

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A B S T R A C T

In this review, we focus on providing basics and examples for each component of the protein therapeutic specifications to interested pharmacists and biopharmaceutical scientists with a goal to strengthen understanding in regulatory science and compliance. Pharmaceutical specifications comprise a list of important quality attributes for testing, references to use for test procedures, and appropriate acceptance criteria for the tests, and they are set up to ensure that when a drug product is administered to a patient, its intended therapeutic benefits and safety can be rendered appropriately. Conformance of drug substance or drug product to the specifications is achieved by testing an article according to the listed tests and analytical methods and obtaining test results that meet the acceptance criteria. Quality attributes are chosen to be tested based on their quality risk, and consideration should be given to the merit of the analytical methods which are associated with the acceptance criteria of the specifications. Acceptance criteria are set forth primarily based on efficacy and safety profiles, with an increasing attention noted for patient-centric specifications. Discussed in this work are related guidelines that support the biopharmaceutical specification setting, how to set the acceptance criteria, and examples of the quality attributes and the analytical methods from 60 articles and 23 pharmacopeial monographs. Outlooks are also explored on process analytical technologies and other orthogonal tools which are on-trend in biopharmaceutical characterization and quality control.

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## 1. Introduction

Protein therapeutics, particularly monoclonal antibody (mAb) therapeutics, are amongst state-of-the-art biomedicines and nowadays represent the fastest growing segment of the pharmaceutical market [1–5]. With higher specificity through the protein's higher-order structure to a target than that of a small molecule drug, they provide less off-target adverse effects and more effectiveness than traditional treatments. While several protein therapeutics gained the blockbuster status in various targeted therapies, their treatment cost is very high [6]. Cost-effective protein therapeutics for patients should be developed with high levels of quality, efficacy, and safety. The quality of therapeutics essentially corresponds to the efficacy and safety. Several reported quality issues have caused significant effects on the patients' health and quality of life and critically

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damaged credibility and reputation of the manufacturers [7–10]. Availability of biosimilar products in the market results in potential savings for public health agencies, insurers, and patients [6,11–14]. However, one of the challenges for a biopharma industry in making good biosimilars is related to their quality issues, i.e., their non-comparable critical quality attributes to those of originators that may result in non-comparable clinical performance [15–20].

As a bridge between consumer protection and drug development, regulatory science is defined by U.S. Food and Drug Administration (FDA) as "the science of developing new tools, standards, and approaches to assess the safety, efficacy, quality, and performance of all FDA-regulated products" [21]. Learning from past and current drug development and understanding the quality, efficacy, and safety of medicines are necessary to develop a study methodology and establish scientific evidence for drug approval. With a perspective on chemistry, manufacturing, and controls (CMC) of protein therapeutics, quality profiles of their drug substances and drug products must be studied and controlled to ensure their efficacy and safety to patients.

### 1.1. What are the biopharmaceutical specifications?

Throughout a drug discovery and development pipeline, design, development, and several control strategies such as in-process controls, good manufacturing practice (GMP) controls, and specifications are keys to ensure the quality and consistency of drug substances (DS) and drug products (DP) [22,23]. Protein therapeutics are classified as "biological medicines" by European Medicines Agency and "biologics or biological products" by US FDA. To distinguish them from other types of biologics, such as conventional vaccines, blood and blood products, and cell and gene therapies, the protein therapeutics produced using biotechnological processes are called "biopharmaceuticals" which can be approved as originators or biosimilars [24]. Protein therapeutic DS comprise a protein which is an active pharmaceutical ingredient (API) in a proper medium, but it is not yet formulated for drug administration to a patient, while DP consists of the protein API and excipients in a formulation and is appropriately packaged so as to enable dosing. Specification is defined to comprise a list of tests, references to test procedures, and appropriate acceptance criteria for the tests [23]. Objective of having the specification along with other control strategies is to ensure that when DP is administered to a patient, its intended therapeutic benefit (efficacy) and safety should be obtained.

According to pharmaceutical inspection convention and pharmaceutical inspection co-operation scheme (PIC/S) GMP for medicinal products, "quality control is that part of good manufacturing practice which is concerned with sampling, specifications and testing, and with the organization, documentation and release procedures which ensure that the necessary and relevant tests are actually carried out …" [25] (Fig. 1A). In countries where GMP is enforced as a law, the specifications are legally-binding for marketed DP. Having the specification is required to set the criteria which DS or DP should conform to Ref. [23], i.e., the results should meet the acceptance criteria when using the listed analytical methods to test the DS or DP. Acceptance criteria of a release specification indicate the quality at the time of release and must be tighter than those in shelf-life specification to have room for changes during storage (a tiered approach for release and shelf-life specifications). Whereas acceptance criteria of a shelf-life specification indicate the quality over a shelf life (Fig. 1B). For biologics, as stated in International Council for Harmonization (ICH) Q5C, "the expiration dating should be based on real-time/real-temperature data", and "since dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process" [26].

Specification settings are also required for intermediate products, pharmaceutical excipients and packing materials in DP manufacturing; however, they are beyond our focus and not mentioned here. Importantly, the acceptance criteria of DS and DP specifications are also applied to accelerated and long-term stability studies, which provide evidence on how the quality of DS and DP vary with time [26]. Data on specification testing, accelerated stability study (with a preliminary shelf-life) and long-term stability testing (to establish an official shelf-life) are required in a common technical dossier (CTD) for drug registration and approval of new biological products and biosimilars [27,28].

### 1.2. Informative/detailed characterization vs. conformance tests

Examples of DS and DP specifications for a mAb are shown in Tables 1 and 2, respectively. Specification testing is "all or none". DS/DP must pass all tests to guarantee its quality. If at least one test

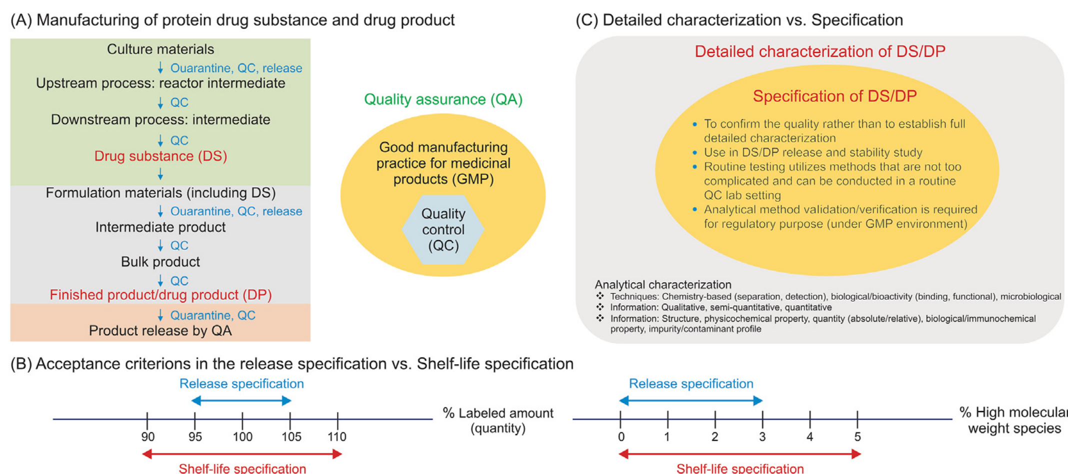


**Fig. 1.** Biopharmaceutical quality control. (A) Schematic diagram of protein therapeutic manufacturing processes and controls under good manufacturing practice (GMP). (B) Diagrams showing acceptance criteria examples for percentage of labeled amount (quantity) and percentage of high molecular weight species in the release and shelf-life specifications. (C) Diagram explaining the detailed characterization vs. the specification of drug substances and drug products.

**Table 1**
Example of an in-house specification for a mAb drug substance.

| Quality attribute | Test procedure | Acceptance criteria |
|---|---|---|
| Appearance | Visual inspection | Complies with in-house description |
| Identification | | |
| pI of intact protein | Imaged capillary isoelectric focusing | Conforms to reference material |
| Tryptic peptide mapping | RPLC-UV | Conforms to reference material |
| Bioidentity | Activity binding ELISA | Conforms to reference material |
| Purity and impurities | | |
| Product-related substances and impurities: Charge variants | | |
| Main peak | IEX-UV | NLT 65.0% |
| Acidic peak | IEX-UV | NMT 15.0% |
| Basic peak | IEX-UV | NMT 20.0% |
| Product-related substances and impurities: Size variants | | |
| Major peak | CE-SDS (non-reduced) | NLT 97.0% |
| Heavy and light chain peaks | CE-SDS (reduced) | NLT 99.0% |
| Monomer | SEC-UV | NLT 98.5% |
| High molecular weight species | SEC-UV | NMT 1.0% |
| Low molecular weight species | SEC-UV | NMT 0.5% |
| Product-related substances and impurities: Post-translational modification (Glycosylation) | | |
| N-glycan profiling | HILIC−Fluorescence detector | Conforms to the limit of each glycan |
| Process-related impurities | | |
| Residual host cell protein | ELISA | NMT 20 ppm (ng/mg) |
| Residual protein A | ELISA | NMT 5 ppm (ng/mg) |
| Residual DNA | qPCR | NMT 40 ppb (pg/mg) |
| Additional process-related impurity | … | … |
| Protein concentration | UV spectroscopy | 90.0−110.0% labeled amount |
| Potency | | |
| Functional assay | Cell-based bioassay | 70.0−130.0% |
| Additional tests | | |
| pH | USP 〈791〉 | 5.5−6.5 |
| Bacterial endotoxins | USP 〈85〉 | NMT 0.20 EU/mg |
| Total microbial count | USP 〈61〉 | NMT 1 CFU/mL |
| Mycoplasma | USP 〈63〉 | Negative result of its nucleic acid |

CE: capillary electrophoresis; ELISA: enzyme-linked immunosorbent assay; HILIC: hydrophilic interaction chromatography; IEX: ion exchange chromatography; NLT: not less than; NMT: not more than; pI: isoelectric point; qPCR: quantitative polymerase chain reaction; RPLC: reversed phase liquid chromatography; SDS: sodium dodecyl sulfate; SEC: size exclusion chromatography; USP: United States Pharmacopoeia; UV: ultraviolet.

**Table 2**
Example of an in-house specification for a monoclonal antibody (mAb) drug product.

| Quality attribute | Test procedure | Acceptance criteria |
|---|---|---|
| Appearance | Visual inspection,<br>USP 〈1〉, USP 〈790〉 | Complies with in-house description |
| Identification | | |
| pI of intact protein | Imaged capillary isoelectric focusing | Conforms to reference material |
| Tryptic peptide mapping | RPLC-UV | Conforms to reference material |
| Bioidentity | Activity binding ELISA | Conforms to reference material |
| Purity and impurities | | |
| Product-related substances and impurities: Charge variants | | |
| Main peak | IEX-UV | NLT 55.0% |
| Acidic peak | IEX-UV | NMT 20.0% |
| Basic peak | IEX-UV | NMT 25.0% |
| Product-related substances and impurities: Size variants | | |
| Major peak | CE-SDS (non-reduced) | NLT 95.0% |
| Heavy and light chain peaks | CE-SDS (reduced) | NLT 98.0% |
| Monomer | SEC-UV | NLT 97.5% |
| High molecular weight species | SEC-UV | NMT 1.5% |
| Low molecular weight species | SEC-UV | NMT 1.0% |
| Protein concentration | UV spectroscopy | 90.0−110.0% labeled amount |
| Potency | | |
| Functional assay | Cell-based Bioassay | 65.0−135.0% |
| Binding assay | Potency ELISA | 70.0−130.0% |
| Other general tests and additional tests for a unique dosage form | | |
| pH | USP 〈791〉 | 5.5−6.5 |
| Osmolality | USP 〈785〉 | 310−370 mOsm/kg |
| Polysorbate 80 (or 20) | RPLC-ELSD | 100−300 μg/mL |
| Particles/vial ≥25 μm | USP 〈787〉, 〈788〉 | NMT 600 particles/vial |
| Particles/vial ≥10 μm | USP 〈787〉, 〈788〉 | NMT 6,000 particles/vial |
| Extractable volume | USP 〈697〉 | NLT 4.0 mL |
| Bacterial endotoxins | USP 〈85〉 | NMT 0.20 EU/mg |
| Sterility | USP 〈71〉 | No microbial growth |

USP: United States Pharmacopoeia; pI: isoelectric point; RPLC: reversed phase liquid chromatography; UV: ultraviolet; ELISA: enzyme-linked immunosorbent assay; IEX: ion exchange chromatography; NLT: not less than; NMT: not more than; CE: capillary electrophoresis; ELISA: enzyme-linked immunosorbent assay; HILIC: hydrophilic interaction chromatography; IEX: ion exchange chromatography; NLT: not less than; NMT: not more than; SDS: sodium dodecyl sulfate; SEC: size exclusion chromatography.

fails, it means the DS/DP does not pass the specification testing. Regarding the scope of tests, informative or detailed characterization of a protein therapeutic candidate is conducted during drug development phase, particularly before establishing a specification. Knowledge gained through thorough characterization of DS and DP provides a strong foundation that allows for a quality by design (QbD) approach for process, formulation, and specification development to mitigate any remaining identified risks [29,30]. Conformance tests of the specification, meanwhile, are performed to confirm the quality rather than to establish full detailed characterization and focus on critical quality attributes found to be useful in ensuring the safety and efficacy (fit for purpose) [23] (Fig. 1C). Some quality attributes that are characterized during a comprehensive study of protein therapeutics or a similarity study of a biosimilar to its reference product may be excluded from the specification if they are not necessary to be a routine quality control (QC) indicator. For instance, analysis of impurities or contaminants which are evidently found not related to/involved in DS and DP (such as post-translational modifications and cross-contaminations that do not occur in that protein therapeutic) should not be included in the specification. Specification testing typically utilizes analytical methods that are not too complicated and can be conducted in a routine QC laboratory setting. For example, determination of high-resolution structure of protein conformation which requires a complicated technique and a costly instrument, such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, is not assigned in the specification. Animal testing is generally not included in the specification either.

Specification tests are performed routinely for a batch release, while periodic/skip or no testing may be allowed for some tests if they are well justified and approved [31], e.g., those of some process-related impurities and contaminants such as elemental and mutagenic impurities [32–35]. Control strategy is based on a risk assessment [36] (See more information in Section 4.1). In the case of higher risks, control by routine specification testing is necessary in addition to other control strategies. For lower risks, control by non-routine characterization can be used to ensure low/non-detected level and consistency along with thorough material sourcing, equipment selection, process control, environment control, and/or another indicative release test [32–35].

### *1.3. Objective and scope of the review*

To provide basic regulatory science of each component of the protein therapeutic specifications, we interpret the quality guideline of the ICH Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological products [23]. ICH Q6B has been adopted since 1999 with its application scope for protein drugs (ranging from insulins to Fc fusions (Table S1 and Fig. S1) to mAbs (Tables 1 and 2, and Fig. S1) and to even larger proteins) and their derivatives (e.g., antibody–drug conjugates (ADCs) (Fig. S1)). Despite being outdated and under a plan for revision [37–39], this guideline is still in a good shape and applicable to most cases of protein therapeutics [39]. Also discussed in this review are other guidelines which support specification setting, examples of quality attributes and analytical methods from a literature review, and brief information about how to set acceptance criteria and statistical consideration [30,31,40–44]. With an advent of regulatory science, those in biopharmaceutical industry should have appropriate scientific and regulatory reasons in choosing analytical tools, setting standards, and designing approaches for the quality control of protein therapeutics. For those in a healthcare system, understanding the specification is necessary for cost-effective procurement of biopharmaceuticals with a focus on their quality, efficacy, and safety.

## 2. Protein therapeutic specifications: list of tests and analytical methods

### *2.1. What tests should be included?*

Conformance of DS or DP to the specification is achieved by testing an article according to the listed tests and analytical methods, and obtaining test results that meet the acceptance criteria. For a compendial article, a list of tests along with analytical methods and limits are shown in an official monograph in a pharmacopoeia. Here, we mainly mention the United States Pharmacopoeia (USP) and the European Pharmacopoeia (Ph. Eur.) for references as they are among the pharmacopoeias which are legally recognized worldwide and with frequent updates on protein therapeutics [45–50]. Examples of USP monographs of protein therapeutics are alteplase, alteplase for injection, epoetin, filgrastim, insulin human and other insulin drug substances, insulin human injection and other insulin drug products, recombinant albumin human, somatropin, and somatropin for injection, and those of Ph. Eur. monographs are erythropoietin, etanercept, filgrastim, filgrastim injection, follitropin, infliximab and the general monograph “monoclonal antibodies for human use” [51–73]. USP general chapter ⟨129⟩ “analytical procedures for recombinant therapeutic monoclonal antibodies” [74] is also mentioned in this review.

Additional tests may be required depending on DS/DP being tested, e.g., process-dependent impurities that are not specified in a compendium and a regulatory statute. There are only a handful number of protein therapeutic monographs in the pharmacopoeia. For non-compendial articles, specification should be set in accordance with the ICH guideline [23], particular pharmacopeial general chapters (listed in Table S2) and/or other regulatory guidance [75–77]. Relevant guidelines that are not mentioned here, particularly for specific types of impurities and contaminants such as host cell proteins, residual DNA, and endotoxins, are also available and helpful for setting the specification.

Table 3 shows the quality attributes that should be included in the list of tests of the protein therapeutic specification, in accordance with the ICH Q6B guideline [23]. Categorically speaking, appearance/description, identity, purity and impurities, and quantity are “universal tests” [22] which are required for all DSs and DPs from small molecules to biologics. Meanwhile, potency, other general tests for DP, and additional tests for DS/DP are considered “specific tests” [22] which depend on specific properties and intended uses of a particular DS/DP. Despite being categorized as specific tests, potency test and tests required for injectable drugs are universal among most protein therapeutics. As stated in the ICH Q8 guideline, a critical quality attribute (CQA) is “a physical, chemical, biological, or microbiological property or characteristic

**Table 3**
Quality attributes that should be included in the protein therapeutic specification, in accordance with the International Council for Harmonization (ICH) Q6B guideline [23]. Quality attributes (A) to (H) are explained in Sections 2.3.1 to 2.3.8.

| Drug substance (DS) | Drug product (DP) |
|---|---|
| Universal tests | |
| A. Appearance and description | A. Appearance and description |
| B. Identity | B. Identity |
| C. Purity and impurities | C. Purity and impurities |
| - Product-related substances | - Product-related substances |
| - Product-related impurities | - Product-related impurities |
| - Process-related impurities | - Process-related impurities |
| D. Quantity | D. Quantity |
| Specific tests | |
| E. Potency | E. Potency |
| F. Additional tests for DS | G. Other general tests for DP |
| | H. Additional tests for a unique dosage form |

that should be within an appropriate limit, range, or distribution to ensure the desired product quality" [78]. To ensure a protein therapeutic is fit for use, the specification with corresponding limits is applied to its CQAs [42]. All quality attributes listed for specification testing should be considered CQAs, with an exception for non-critical quality attributes (with lower risks than CQAs) which are not related to efficacy and safety, e.g., those for consumer satisfaction or other purposes, and non CQAs which are simple and useful to monitor and still have an impact on efficacy and/or safety.

### 2.2. What analytical methods should be used?

How each quality attribute is tested is governed by the analytical method being used in the test. Consideration should be given to figures of merit of the method, such as specificity and sensitivity, which are associated with the acceptance criteria of the specification. According to the PIC/S GMP [25], analytical methods being used in a batch analysis as per the specification must pass analytical instrument qualification [79] and analytical method validation (AMV) [80,81] prior to implementation in a routine QC. AMV is the process to demonstrate and document that the test method has performance characteristics that meet appropriate standard for reliability and therefore suitable for its intended use [80,81]. The guideline ICH Q2 Validation of Analytical Procedures [80,82] and the general chapter 〈1225〉 Validation of Compendial Procedures [81] are suggested for AMV. Examples of AMV for the analysis of protein biopharmaceutical are referenced [83–87]. As stated in the revised guideline, ICH Q2(R2) [82], typical performance characteristics of the analytical procedure to be evaluated in AMV are specificity/selectivity, working range (linear or nonlinear response, and lower range limits—detection and quantitation limit), accuracy, and precision (repeatability and intermediate precision). Analytical methods for different types of measured product attributes have different performance characteristics and criteria, as suggested in the guideline. Other characteristics, such as robustness, reproducibility, and system suitability, may be evaluated in AMV on a case-by-case basis. Acceptance criteria of the performance characteristics are not explicitly stated in the ICH guidelines. Some pharmacopeial general chapters (those with analytical performance requirements) and Association of Official Analytical Collaboration (AOAC)'s guidelines for standard method performance requirements [88] can be referred in order to set the criteria.

For certain types of tests, there are additional guidelines to support their AMV; for example, validation of biological assays (i.e., relative potency bioassays such as binding, cell-based, and animal-based assays) is guided by the general chapter 〈1033〉 Biological Assay Validation [89]. Analytical method verification [90], possibly with the smaller number of experiments, can be applied instead of AMV for compendial methods stated in the pharmacopoeia. This is because, unlike in-house developed methods, the official methods were thoroughly validated by monograph developers.

Here, the term "method validation" is for analytical methods used in the analysis of protein pharmaceuticals—DSs and DPs. Do not be confused this term with the "bioanalytical method validation" which is for analytical methods used in the bioanalysis, i.e., analysis of biological samples such as blood and tissues [91]. Bioanalysis deals with a low-level analyte in a complex biological matrix; thus, the method's analytical performance criteria are not comparable with those of the method for a higher-level analyte in a simpler matrix. The new ICH guideline for bioanalytical method validation (ICH M10) [92] lists the performance characteristics of chromatographic and ligand-binding assays (such as specificity, selectivity, matrix effect, calibration curve and range, accuracy and precision, carry-over, dilution integrity, and stability) and their acceptance criteria. Some bioanalyses of protein therapeutic are referred to as examples of the bioanalytical method validation [93–96].

### 2.3. What quality attributes of protein therapeutic drug substance/drug product and analytical methods should be included?

Listed below are quality attributes of DS and DP which are suggested in the guideline ICH Q6B [23] (Table 3) and should be included in the specifications. Examples of protein therapeutic's CQAs and proposed analytical methods for testing are shown in Table S1. A variety of analytical methods that are applicable for testing each quality attribute are listed in Table S2. Review papers and books on protein therapeutic's CQAs and their analytical assessments are suggested as further readings [97–110].

Examples of utilizing certain analytical methods in testing the CQAs of protein therapeutics, from the literature review of articles published over a recent decade, are shown in this review. There are 60 original research articles of which 52 on mAbs, 3 on Fc fusions, 3 on hormones, 1 on combination of mAbs, and 1 on ADC [111–170] (Table 4), and 23 pharmacopeial monographs of which 2 on mAbs, 1 on Fc fusions, 17 on hormones, 2 on enzymes, and 1 on other protein [51–73] (Table 5). Most of the selected articles focus on analytical characterization of originators and biosimilars, while others focus on orthogonal analytical methods for investigating certain CQAs of protein therapeutics. Tables 4 and 5 show analytical techniques used in characterizing CQAs of those protein therapeutics. Because DS and DP specifications are confidential proprietary data of manufacturers, we do not have access to their information. Several tests in the literature that are mentioned in Tables 4 and S2 were conducted during early-stage drug development, comparability or biosimilar studies which represent informative/detailed characterization rather than conformance tests used in the specification. Despite this, the examples are deemed helpful for a reader to know what analytical techniques can be used to characterize each quality attribute of protein therapeutics and, with a regulatory science aspect, which ones are suitable for a routine QC. GMP conformance tests used in the pharmacopeial specifications are shown in Table 5.

#### 2.3.1. Appearance and description (A)

Organoleptic appearance of DS and DP, such as physical state (powder or liquid), color, and clarity of solution, can be visually inspected [23]. Any change in these characteristics indicates something wrong with drug quality which may affect efficacy and safety. The color of a protein therapeutic solution, suspension, or powder is a critical quality attribute. Color variation at time of DS/DP release and during stability studies indicates the presence of degradation products, e.g., those resulting from oxidation of Trp [97] or other impurities/contaminants [171,172]. Meanwhile, protein aggregation can lead to cloudiness of a solution. For QC, this test should be performed before other tests listed in the specification because of its simplicity as it can be performed solely by organoleptic observation in most cases, with no use of analytical methods (Table S2). If the test fails, there is no need to perform further tests, and an out-of-specification investigation is needed.

Every unit of parenteral products should be thoroughly inspected for visible particulate matters [173–176] while instrumental methods are needed for determination of subvisible particulate matters [177] (Section 2.3.8). Different microscopic methods with image analyses are used for subvisible (protein) particle analysis [178,179]. Image analysis is also used in appearance/description inspection of solid dosage forms [180]. However, none is reported for inspection of dosage forms containing protein therapeutics.

**Table 4**
Literature review of analytical techniques used in characterizing the critical quality attributes (CQAs) of certain protein therapeutics. Asterisk (*) denotes the analytical technique commonly used in both detailed characterization and specification.

| List of tests/quality attributes | Analytical techniques | References |
|---|---|---|
| Identity (B) | | |
| Overall structure | | |
| Intact mass | ESI-MS | [111] |
| | LC-ESI-MS* | [112–146] |
| | MALDI-MS | [132] |
| Subunit mass | LC-ESI-MS | [112–119,121,122,124–129,131–133,135–139,141–151] |
| Isoelectric point (pI) | See Charge variants – IEF | |
| UV spectrum/absorptivity | UV spectroscopy* | [131,152] |
| Primary structure | | |
| Peptide mapping | LC-ESI-MS, MS/MS | [111–131,133–140,143–161] |
| | LC-UV* | [113,114,116,117,120,123,124,126,129,131,133–138,142,145,147,150–152,157,159–162] |
| | MALDI-MS, MS/MS | [141] |
| Terminal amino acid sequence | LC-ESI-MS, MS/MS | [125,127,129,136,139,144,149,160] |
| | Edman sequencing | [111] |
| Amino acid composition | Amino acid analysis | [138] |
| Higher-order structure | | |
| Secondary structure | Far-UV CD spectroscopy | [111,112,116,118,119,121–125,127–131,135–137,139–142,144,145,147,150,152,153,158,160,163] |
| | FT-IR spectroscopy | [111,113,114,116,118–123,125–131,135,138–140,142,143,152] |
| Tertiary structure | Near-UV CD spectroscopy | [111–114,116,119–131,135,137–143,147,150,152,153,160] |
| | Fluorescence spectroscopy | [111,116,118,119,121,125,128,129,131,136,139,141,142,145,153,165] |
| Thermal stability | DSC | [111–114,116,119,120,122–131,134–136,138–140,142,143,145,147,150,152,153,158] |
| | DSF | [144] |
| Gas-phase mobility | IM-MS | [132,153] |
| Site-specific conformation | HDX-MS | [119,125,130,135,140] |
| | CL-MS | [163] |
| Bioidentity | See Potency – Bioassays | |
| Purity and impurities (C) | | |
| Product-related substances and impurities: Charge variants | | |
| Acidic species | IEX-UV* | [111–115,117,119,121–128,130,131,133,135,136,138–145,148–150,152,154–156,158,160,162,164,165] |
| Main species | | |
| Basic species | IEX-MS | [154,164] |
| | CIEF electrophoresis – UV* | [113,115,117,126,131,136,138,140,142–144,150,155,160] |
| | Imaged CIEF* | [117,119,122,124,125,127,128,130,139,145,147,148,151,166] |
| | IEF gel electrophoresis* | [111,112,123,131,152] |
| Product-related substances and impurities: Size variants | SEC-UV* | [111–127,130,131,134–136,139–145,147,149–153,158,162,165,166] |
| High molecular weight species | SEC-MALS | [112–114,119,120,123–126,130,131,138–140,143–145,150,152] |
| Low molecular weight species | Analytical ultracentrifugation | [112–114,116,119,120,123,125,126,128,130,136,138–141,147,152] |
| | FFF | [111,113,135] |
| | CE-SDS* | [111–115,117,119,122,124–127,130,131,134–136,138–140,142,143,145,149,150,152,158,166] |
| | SDS-PAGE* | [121,133,141,153,162] |
| | Gel-on-a-chip electrophoresis | [144,147] |
| | DLS | [113,121,128,136,138,139,144,145,165] |
| | IM-MS | [153] |
| Product-related substances and impurities: PTMs | | |
| Protein: intact mass | See Identity – Intact mass | |
| Protein: hydrophobic variants | RPLC-UV* | [116,118,127,140,142] |
| Protein: free thiol | Reagent test (e.g., Ellman's assay, Thiol Fluorescent Detection Kit, Measure-IT™ Thiol Assay Kit) | [112,119,122–124,128,129,131,135,139,142,145,150,152] |
| Subunit: subunit mass | See Identity – Subunit mass | |
| Subunit: glycosylation | HILIC – MS | [140,149,152,167] |
| Peptide: peptide mapping (PTMs such as oxidation, deamidation, C-terminal Lys truncation, glycosylation, etc.) | LC-ESI-MS, MS/MS | [111–113,115,117–119,121–137,139–141,143–147,149,152–157,159,160,164] |
| | LC-UV | [134–136,145,155,158,159] |
| Peptide: disulfide profiling | LC-ESI-MS, MS/MS | [113,115–119,122–126,128–131,135,136,139,140,142,144,145,147,150,152] |
| | LC-UV | [113,124,136,145,150,158] |
| Released glycan profiling | HILIC – Fluorescence detector* | [112–115,117,119,120,122–129,131,135–139,142–146,150,152,156,157,159,162,169,170] |
| | HILIC-MS | [113,114,117,121,126,129,131,132,138,156,157] |
| | RPLC – Fluorescence detector | [122,140] |
| | CE – Fluorescence detector | [117,133,168] |
| Monosaccharide profiling | IEX – Pulsed amperometric detector* | [112,123,128,136] |
| | RPLC – Fluorescence detector* | [111,117,128,145,152,157] |
| | HILIC – Fluorescence detector | [145] |
| Process-related impurities | | |

**Table 4** (*continued*)

| List of tests/quality attributes | Analytical techniques | References |
|---|---|---|
| Host cell proteins | ELISA* | [111,113,128,138,144,145,152] |
| | 2D LC-MS | [113,138] |
| | 2D gel electrophoresis | [113,138] |
| Residual protein A | ELISA* | [113,128,144,145,152] |
| Residual DNA | qPCR* | [113,122,128,138,144,145,152] |
| Quantity/protein content (D) | UV* | [111,113,119–121,123,126,138–140,143–145,150,158] |
| | HPLC-UV* | [116,118] |
| | Amino acid analysis | [144] |
| Potency/biological activity (E) Binding tests Functional tests | ELISA* | [111–115,117,119,120,122,123,125–130,136–140,142,143,145,147,149,150,152,153,160,169] |
| | SPR | [111–114,116,117,119,121–129,131,133–137,139,140,142,143,145,147,152,160,170] |
| | BLI | [117,127,149,150,153] |
| | Bead-based assay (e.g., AlphaScreen®, AlphaLISA®) | [113,114,116,119,120,125,126,169,170] |
| | Affinity chromatography | [128] |
| | FRET | [119,125,166,169,170] |
| | FACS | [111,119,121–125,131,136,145,147,170] |
| | Reporter assay | [117,125,126,129,130,136,139,145,150,160,170] |
| | Cell-based assay* | [111–139,141,143,145,147,149–152,157,158,160–162,166,169,170] |

Note: Asterisk (*) denotes the analytical technique commonly used in both detailed characterization and specification. Tests for other process-related impurities, other general tests and additional tests are not listed in this table.
2D: two-dimensional; BLI: bio-layer interferometry; CD: circular dichroism; CE: capillary electrophoresis; CIEF: capillary isoelectric focusing; CL: covalent labeling; CZE: capillary zone electrophoresis; DLS: dynamic light scattering; DSC: differential scanning calorimetry; ELISA: enzyme-linked immunosorbent assay; ESI: electrospray ionization; FACS: fluorescence-activated cell sorting; FFF: field-flow fractionation; FRET: fluorescence resonance energy transfer; FT: Fourier transform; HDX: hydrogen–deuterium exchange; HIC: hydrophobic interaction chromatography; HILIC: hydrophilic interaction chromatography; IEF: isoelectric focusing; IEX: ion exchange chromatography; IM-MS: ion mobility–mass spectrometry; IR: infrared; LC: liquid chromatography; MALDI: matrix-assisted laser desorption ionization; MALS: multi-angle light scattering; MS: mass spectrometry; MS/MS: tandem mass spectrometry; PAGE: polyacrylamide gel electrophoresis; PTM: post-translational modification; qPCR: quantitative polymerase chain reaction; RPLC: reversed phase liquid chromatography; SDS: sodium dodecyl sulfate; SEC: size exclusion chromatography; SPR: surface plasmon resonance; UV: ultraviolet.

### 2.3.2. *Identity (B)*

Primary and higher-order structures are an amino acid sequence and a unique fold of the protein, respectively, and these quality attributes can indicate the protein identity. As a qualitative analysis, the identity test should be highly specific to the protein therapeutic in DS and DP [23]. Table S2 outlines analytical methods that are applicable to the identification. Identification test in the specification does not mean a structure elucidation to determine a high-resolution protein structure like that obtained from X-ray crystallography and NMR. A comparative test (a standard/reference vs. a sample) is usually performed to ensure the identity. Otherwise, a specific characteristic of an analyte should be determined for the identification, e.g., peptide masses and sequencing spectra. More than one identity tests are used to get complementary information for extra confirmation [23].

Correct identity (sequence and folding) of the protein therapeutic is required to ensure its efficacy and safety [181]. Manufacturing of DP using a wrong API may happen. Even though the protein DS is already identified, an active ingredient in DP must be identified as well using the same and/or complementary methods, but maybe with fewer tests.

*2.3.2.1. Primary structure.* From the literature review (see references in Table 4), the primary structure of protein therapeutics is mainly characterized through intact and/or subunit mass measurement using LC-MS technique [112–151] and peptide mapping using a bottom-up LC-MS, with MS/MS for peptide sequencing [111–131,133–140,143–161]. Modern MS can accurately measure the molecular weight of an IgG about 150 kDa with the accuracy of ≤2 Da [113]. Information about protein and its PTMs (modified protein) from a mass spectrum is the protein ion's mass-to-charge ratio (*m/z*) with a charge-state distribution, on which deconvolution can be performed to calculate the molecular mass of protein (neutral mass in Da). MS resolution should be concerned for accuracy and precision of *m/z* measurement, particularly with ability to distinguish *m/z* of different charge states and modifications (PTMs).

At the protein/subunit level, reversed-phase liquid chromatography (RPLC) is mostly used in the separation due to a physicochemical nature of proteins/peptides that are relatively hydrophobic. In official monographs of several protein therapeutics, RPLC-UV is used for the intact protein analysis (via retention time comparison with a standard) [51–62]. Intact mass measurement using MS is also mentioned as another official test [63]. Other compendial methods for identifying the protein's primary structure are isoelectric focusing (IEF) [64–66] for isoelectric point identity, ion-exchange chromatography (IEX) [66] for charge identity, capillary zone electrophoresis (CZE) [67] for charge and size identities, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) [64,67] and size exclusion chromatography (SEC) [64,65] for size identity, immunoblotting [67] for specific sequence identity, etc. (Table 5).

At the peptide level, RPLC-UV is used for the peptide mapping according to several official monographs [51,53,55,57,59,62–65,67–72]. Peptide mapping is suggested in Ph. Eur. for the quality control of mAbs for human use [66] (Table 5). LC-UV is less complicated and much cheaper than the MS-based method, but it is with a lower specificity and need a comparison with a reference. As a comparative identity test, a test sample is digested and assayed in parallel with the reference using LC-UV. To achieve complete digestion, certain proteins should undergo deglycosylation and/or disulfide reduction prior to the digestion. Unique fingerprint of LC-UV peptide map can provide enough specificity in the primary structure analysis than that of using the protein's high-performance liquid chromatography (HPLC) retention time alone [182,183].

*2.3.2.2. Higher-order structure (HOS).* A variety of analytical techniques can characterize the HOS of protein therapeutics (Table 4), and they are among decision-making tools during drug development as HOS has a critical role in maintaining the stability and

**Table 5**
Compendial analytical techniques/test procedures in the United States Pharmacopoeia (USP) and European Pharmacopoeia (Ph. Eur.) specifications of certain protein therapeutics.

| List of tests/quality attributes | Compendial analytical techniques/test procedures | References |
|---|---|---|
| A. Appearance and description | Visual/organoleptic inspection,<br>Instrumental measurement of color | USP, Ph. Eur. |
| B. Identity | | |
| Overall structure | | |
| Retention time | LC-UV | [51–62] |
| Intact mass | LC-ESI-MS | [63] |
| Isoelectric point (pI) | IEF | [64–66] |
| Charge identity | IEX-UV | [66] |
| Charge and size identities | CZE-UV | [67] |
| Size identity | SEC-UV | [64,65] |
| | SDS-PAGE | [64,67] |
| Specific sequence identity | Immunoblotting | [67] |
| Primary structure | | [51,53,55,57,59,62–72] |
| Peptide mapping | LC-UV | |
| Higher-order structure | | [51,53,55,57,59,61,62,64–73] |
| Bioidentity | Bioassays (qualitative assays) | |
| Purity and impurities | | |
| Product-related substances and impurities: Charge variants | IEX-UV, CIEF | [66,72] |
| | IEF gel electrophoresis | [51,64,68,72,73] |
| Product-related substances and impurities: Size variants | SEC-UV, CE-SDS-UV | [52–74] |
| | SDS-PAGE | [51,64,65,70,71] |
| Product-related substances and impurities: PTMs | | |
| Protein: hydrophobic variants | RPLC-UV, HIC-UV, HILIC-UV | [51–55,57–59,61,62,64–66,71,73] |
| Released glycan profiling | HILIC – Fluorescence detector,<br>IEX – Fluorescence detector | [65,67,68,71,72,74] |
| | RPLC – Fluorescence detector | [71] |
| Monosaccharide profiling | IEX – Pulsed amperometric detector | [74] |
| | Reagent test | [67] |
| Quantity/protein content | UV | [51,63,66–72] |
| | RPLC-UV | [52–62] |
| | HPLC (other type) – UV | [64,65,73] |
| | SDS-PAGE | [63] |
| Potency/biological activity | Bioassays (quantitative *in vitro* and *in vivo* assays) | [51,53,55,57,59,61,62,64–73] |

Note: Tests for process-related impurities, other general tests and additional tests are not listed in this table.
CE: capillary electrophoresis; CIEF: capillary isoelectric focusing; CZE: capillary zone electrophoresis; ESI: electrospray ionization; HIC: hydrophobic interaction chromatography; HILIC: hydrophilic interaction chromatography; IEF: isoelectric focusing; IEX: ion exchange chromatography; LC: liquid chromatography; MS: mass spectrometry; PAGE: polyacrylamide gel electrophoresis; RPLC: reversed phase liquid chromatography; SDS: sodium dodecyl sulfate; SEC: size exclusion chromatography; UV: ultraviolet; Ph. Eur. European Pharmacopoeia; USP: United States Pharmacopoeia.

function of protein therapeutics [184–186]. Far-UV circular dichroism (CD) [111,112,116,118,119,121–125,127–131,135–137, 139–142,144,145,147,150,152,153,158,160,163] and mid-infrared (IR) spectroscopy [111,113,114,116,118–123,125–131,135,138–140, 142,143,152] are often used in characterizing the secondary structure, while near-UV CD [111–114,116,119–131,135,137–143,147,150, 152,153,160] and intrinsic fluorescence spectroscopy [111,116,118, 119,121,125,128,129,131,136,139,141,142,145,153,165] are applicable for the tertiary structure characterization. Differential scanning calorimetry (DSC) is often used in determining the thermal stability of protein therapeutics [111–114,116,119,120,122–131,134–136, 138–140,142,143,145,147,150,152,153,158]. Techniques with higher structural resolution, such as NMR spectroscopy [187–189], hydrogen–deuterium exchange (HDX)-MS [119,125,130,135,140, 190–192], and covalent labeling (CL)-MS [163,193], can be used to gain the site-specific information of HOS and are more sensitive to a structural change.

However, these physicochemical methods are not routinely applied to the identity test of HOS in the specification, perhaps mainly due to poor correlation to biological function. Many techniques require an expensive instrument, expertise, a complicated procedure, and data analysis. High-resolution NMR is not applicable to mAbs (and similar-sized proteins) due to the size limitations. While the stability and comparability studies employing NMR are certainly feasible, they have to rely on chemometric approaches [187,189]. In official monographs, bioactivity outcomes from bioassays/potency assays are used to indicate the bioidentity of protein [51,53,55,57,59,61,62,64–73] (Table 5).

### *2.3.3. Purity and impurities (C)*

In contrast to small-molecule drugs, most protein therapeutics exist in many variants due to heterogeneity of the protein during manufacture and/or storage of DS or DP. Common variants of the therapeutic protein are size variants, charge variants, and post-translational modification/PTM variants. ICH Q6B classifies the variants that are related to a therapeutic molecule into product-related substances and impurities. "Product-related substances" are defined as molecular variants of the desired therapeutic which are active and with no detrimental effect on the safety and efficacy [23,97], e.g., certain charge and glycan variants. Meanwhile, "product-related impurities" are molecular variants whose efficacy and/or safety profiles are not comparable to those of the desired therapeutic [23,97]. Examples are precursors (preproteins), high-molecular weight species, and degradation products.

Impurities are described as any component that is not the chemical entity defined for a raw material and a formulation ingredient, and it has no therapeutic benefit and may pose risks for toxicity and/or adverse effect on drug stability [194]. In addition to the product-related impurities, "process-related impurities" derive from manufacturing processes [23,97]. Examples are cell substrates (e.g., host cell proteins (HCPs), host cell DNA), cell culture (e.g., inducers, antibiotics or media components) and down-stream processing (e.g., protein A). Fig. 2 illustrates product-related substances and impurities, and process-related impurities arising during the manufacture of protein therapeutic DS and DP. As per ICH Q6B [23], microbiological and other contaminants are categorized as "process-related contaminants". We explain about them in Sections 2.3.6 and 2.3.8.

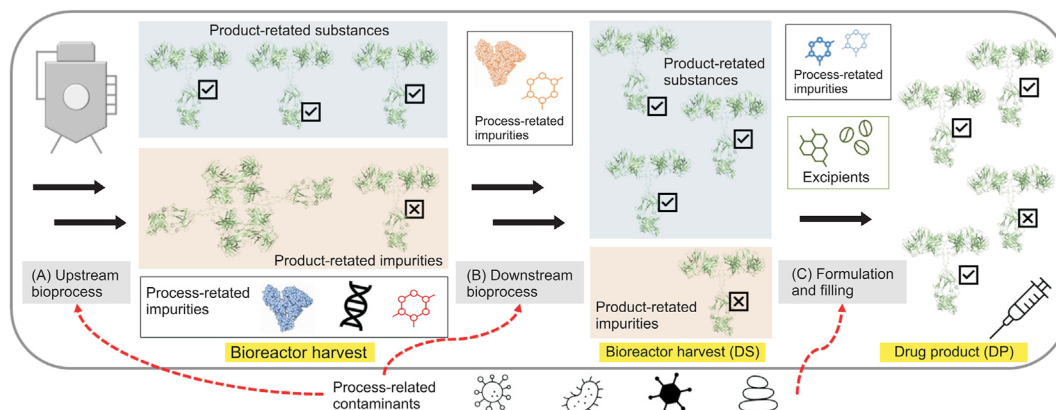


**Fig. 2.** Product-related substances and impurities and process-related impurities and contaminants. They arise during a manufacture of the protein therapeutic drug substance (DS) and drug product (DP) in different processes: (A) upstream and (B) downstream bioprocesses, and (C) formulation and filling. Examples of "product-related substances" are charge isoforms and glycoforms which have no detrimental effect on safety and efficacy. On the other hand, high-molecular weight species, certain degradation products and post-translational modifications that can lead to impaired efficacy or safety profiles are considered "product-related impurities". "Process-related impurities" are derived from manufacturing processes such as host cell proteins, host cell DNA, and cell culture substrates. Meanwhile, "process-related contaminants" such as microbial species and dust particles which are not intended to be part of the manufacturing process but may contaminate DS and/or DP.

Separation of the desired protein species from product-related substances and from impurities in DS or DP is a focus for a choice and optimization of analytical procedures [23]. Chemistry-based analytical techniques (Table S2) for testing purity and impurities should have enough specificity and sensitivity to indicate a small change, i.e., being stability-indicating. In addition, as the impurity in DS/DP is present in a small amount (vs. API amount), the technique used in an impurity test should be sensitive enough, particularly for trace impurities, such as residual host cell proteins. The purity and impurities are usually determined using a combination of methods as it is difficult to determine the absolute purity of protein therapeutics, and the analytical results are method-dependent [23], specifically the size, charge, and PTM variants [97]. Testing of certain product-related and process-related impurities in DP is not necessary if their profiles are the same as that in DS. For process-related impurities that are introduced and/or product-related substances and impurities (degradation products) that are formed during the production and/or storage of DP, the levels of these impurities in DP should be monitored [23]. Without sufficient data to support during an early-phase manufacture, related substances and impurities of protein therapeutics are the most difficult quality attributes to be established in the specification. Informative characterization of a protein therapeutic is needed to thoroughly study impurity profiles from early-to late-stage development and, along with results from nonclinical and clinical studies, to ultimately decide what impurities to be included in the specification and at how much levels as acceptance criteria [44,97].

*2.3.3.1. Size variants.* SEC [111–127,130,131,134–136,138–145,147,149–153,158,162,165,166] and CE-SDS [111–115,117,119,122,124–127,130,131,134–136,138–140,142,143,145,149,150,152,158,166] are widely used in the size variant characterization (Table 4). In the USP general chapter on analytical procedures for recombinant therapeutic mAbs, and USP and Ph. Eur. monographs of protein therapeutics, SEC-UV and CE-SDS-UV are used as analytical methods to determine the product-related substances and impurities (high molecular weight (HMW) and low molecular weight (LMW) protein impurities) [52–74]. SDS-PAGE is still mentioned in some monographs [51,64,65,70,71] (Table 5). SEC and CE-SDS have acceptable size resolution and reliable results about protein size, and these techniques can be routinely performed at affordable cost. SEC is commonly used for the analysis of HMW species as it is conducted in native-like conditions which help preserve non-covalent protein complexes. Meanwhile, CE-SDS, which has higher size resolution, is suitable for the analysis of LMW species. HMW species, which include protein oligomers and aggregates resulting from misfolding or certain PTMs, are the most observed product-related impurities, and close monitoring is required due to immunogenicity concerns [181,195]. Meanwhile, LMW species result from protein truncation by chemical degradation or proteolysis [97,98,104,107].

*2.3.3.2. Charge variants.* IEX [111–115,117,119,121–128,130,131,133,135,136,138–145,148–150,152,154–156,158,160,162,164,165] and IEF electrophoresis [111–113,115,117,119,122–128,130,131,136,138–140,142–145,147,148,150–152,155,160,166] are commonly used in characterization of the charge variants (acidic and basic species), which are considered the product-related substances (i.e., isoforms) and impurities (Table 4). IEX and capillary isoelectric focusing (CIEF) are mentioned in some official monographs [66,72]. IEF gel electrophoresis is also mentioned as a pharmacopeial method for characterizing isoform distribution and charge-variant impurities [51,64,68,72,73] (Table 5). A major peak and other acidic and basic peaks are a general charge variant profile of mAbs [97]. Acidic variants have less amine groups and/or more carboxylic groups (such as deamidation, sialic acid, and N-terminal pyroglutamate formation), while basic variants have those in opposite (such as C-terminal Lys). Most of the charge variants result from PTM of therapeutic proteins, and this CQA quite overlaps with a PTM quality attribute. Hence, the charge variant analysis can be replaced with the PTM analysis, and *vice versa*, if well justified. Based on efficacy and safety data of protein therapeutics, specific charge variant/PTM species are considered "product-related impurities", while others are "product-related substances". Review articles regarding the efficacy and safety of product-related substances and impurities for certain (group of) protein therapeutics are available [20,97,98,104,196–198].

*2.3.3.3. Post-translational modification variants.* Most of the PTM characterizations are performed at the peptide level as the chromatographic separation of modified peptides is more efficient than that of protein subunit or intact proteins with modifications [111–113,115–119,121–137,139–147,149,150,152–160,164]

(Table 4). In many studies, multi-attribute method (MAM) is an LC-MS-based peptide mapping method for characterizing heterogeneity of protein therapeutics [156,199,200]. The benefit of simultaneously measuring several site-specific protein modifications is offered by MAM to monitor different CQAs in a single LC-MS run [199,200]. MAM is with high specificity due to the bottom-up nature of the approach, where the protein is enzymatically digested to smaller peptides prior to LC-MS analysis, which gives information about *m/z* and retention time of the peptide for PTMs such as oxidation, deamidation, C-terminal Lys truncation, glycosylation, etc. During a survey experiment, MS/MS is performed to identify sequence and PTM site of each peptide. Hence, it provides much more detailed information about individual protein modifications than the conventional methods, e.g., IEX and CE-SDS for the analysis of intact protein or subunits, and it is being proposed to be used in a routine QC work [199,200]. For disulfide linkage mapping, peptide mapping either by LC-MS or LC-UV under non-reducing conditions is used to characterize disulfide bonds [113,115−119, 122−126,128−131,135,136,139,140,142,144,145,147,150,152,158]. Reagent test (Cys labeling reaction) can be conducted at the intact protein level to characterize free thiol groups and disulfide bonds [112,119,122−124,128,129,131,135,139,142,145,150,152] (Table 4). In many official monographs, RPLC, and in some cases hydrophobic interaction chromatography (HIC) and hydrophilic interaction chromatography (HILIC), are mentioned for the determination of related substances and impurities (including PTMs) based on different hydrophobicity/hydrophilicity at the intact protein level [51−55,57−59,61,62,64−66,71,73] (Table 5).

For glycosylation profiling, HILIC is often selected for the analysis of glycans/oligosaccharides released from protein therapeutics [112−115,117,119−129,131,132,135−139,142−146,150,152,156,157, 159,162,169,170] (Table 4). At the glycan level, the physicochemical difference of glycan types is more significant than that at the protein and peptide levels, which allows better LC separation (higher chromatographic resolution) for the characterization. Because the glycan does not have a chromophore for UV-Vis or fluorescence detection, fluorescent labeling is performed prior to HILIC so that the labeled glycans can be detected by a fluorescence detector. In the USP general chapters on analytical procedures for recombinant therapeutic mAbs and USP and Ph. Eur. monographs of protein therapeutics, HILIC and IEX with fluorescence detection are used as an analytical method for the N-glycan profiling [65,67,68,71,72,74]. In addition, monosaccharide profiling for sialic acid analysis of mAbs can be performed using a reagent test [67], RPLC with a fluorescence detector [71], or IEX with an amperometric detector which is label-free [74] (Table 5). A variety of analytical techniques for monitoring therapeutic protein glycosylation are mentioned in the USP general chapters on glycoprotein and glycan analysis [201], oligosaccharide analysis [202], and monosaccharide analysis [203], and the Ph. Eur. general chapter on glycan analysis of glycoproteins [204] where glycan profiling is suggested for the quality control of mAbs for human use [66].

References on PTMs relevant to biopharmaceuticals are suggested as comprehensive resources [97,98,104,107]. PTMs with safety or efficacy concerns and those with less significant impact are listed and summarized in Table S3 [97,98,104,107].

*2.3.3.4. Process-related impurities.* To monitor these impurities in DS, enzyme-linked immunosorbent assay (ELISA) is often used to characterize residual HCPs and residual protein A [111,113,128,138,144,145,152], while residual DNA can be monitored using quantitative polymerase chain reaction (qPCR) [113,122,128,138,144,145,152] (Table 4). These methods along with others are mentioned in the general chapters on residual HCP measurement [205] and residual DNA testing [206]. USP and Ph. Eur. do not provide compendial methods and acceptance criteria for these measurements as these impurities are process specific, and validated methods should be applied to determine their levels, with the exception for residual DNA from common hosts (*E. coli* and Chinese hamster ovary cells) for which the compendial qPCR methods to measure these impurities are available [206]. Some HCPs are difficult to remove during the downstream bioprocess and considered "problematic" for the safety profile as they may be immunologic or biologically active [207]. Analytical methods that are applicable to HCP measurement are discussed in detail in these review papers [207−210].

The overall impurity profile of the protein therapeutic DS/DP consists of degradation products of the protein and other product-related impurities, and process-dependent impurities. The latter depends on an expression system, excipients, equipment, and a container-closure system being used in manufacturing. Most of the process-dependent impurities are process-related impurities while some are product-related impurities, such as a remaining protein precursor. These impurities may not be listed in a monograph of the official DS/DP articles because they are uncommon, and a manufacturer has responsibility to perform tests on these impurities to ensure the quality of protein therapeutics. Although elemental and mutagenic impurities and residual solvents are not common in the production of protein therapeutics, they may be present in some processes and pharmaceutical excipients [32−35,211,212]. Risk assessments are needed, and a test must be performed if there is a risk of having each of these impurities in DS/DP to evaluate whether the test should be included in the specification [32−35,211].

*2.3.4. Quantity (D)*

An appropriate chemistry-based assay (Table S2) should be used to determine the quantity of API in DS/DP, based on protein content (mass) [23], with an objective to monitor if the content of API in DS (as percentage purity) or the content of API in DP (as percentage of the total protein content stated on the label) is in an acceptable range (not too low or high) to ensure efficacy and safety. Analytical methods used in the quantitative analysis should be specific, sensitive, accurate, and precise enough for stability indicating. Otherwise, if a quantitation method in the specification is not specific and/or sensitive enough, other methods used for determining purity/impurities and potency should be stability-indicating enough. In cases where therapeutic potency (in unit) is used to indicate the amount of protein, the quantity can be inferred from a potency test, and determination of mass quantity may not be needed [23].

The quantity of protein therapeutics is often determined using UV spectroscopy (total protein concentration measurement) [111,113,119−121,123,126,138−140,143−145,150,158] or LC-UV [116,118] (Table 4). LC provides the separation of species that reduces interference in a measurement and improves the specificity of method. In official monographs [52−62], RPLC-UV is commonly used for the protein which is quite homogeneous, i.e., whose PTM and charge variants are limited. This is perhaps to have almost all species of therapeutic protein eluted in a single or a few peak(s) for LC-UV quantitation. For protein therapeutics whose species are more heterogeneous [51,63,67−72], such as mAbs, their quantity can be determined as the total protein concentration (e.g., using UV spectrophotometry), and a potency assay should be conducted to give information about the quantity of an active protein. Other official tests for protein quantity determination are SDS-PAGE with staining [63], SEC-UV [65], and other LC techniques [64,73]. Protein quantity is one of the CQAs suggested for the pharmacopeial quality control of mAbs for human use [66] (Table 5).

*2.3.5. Potency (E)*

Quantitative assessment of potency is a required test in the protein therapeutic specification which is conducted using a validated biological assay/bioassay (Table S2) to measure the biological activity (in unit) [23,213]. Bioassay method should be stability-indicating, i.e., specific and sensitive enough to indicate small change. For complex molecules like therapeutic proteins, the potency assay is also performed to give information regarding the HOS which chemistry-based analytical tools may be unable to confirm well [23].

Different bioassays can be used to determine the potency as binding or functional activities [111−143,145,147,149−153,157,158,160−162,166,169,170] (Table 4). ELISA and surface plasmon resonance (SPR) are techniques commonly used in binding assay. However, SPR is not a common technique for routine quality control due to a high instrument cost. It should be noted that higher binding affinity does not always mean a more potent antibody therapeutic [196]. Cell-based and some animal-based tests are used as functional assays to reflect a mechanism of action of the protein therapeutic, and the functional assay should be a part of the specification [214,215]. With an exception in cases where target binding is simply the mechanism of action, binding assay may be sufficient for the potency determination [214,215]. The use of binding assay vs. cell-based assay as the potency release test should be rationalized by experimental data [215]. In official monographs, potency test is usually required as a part of the specification of protein therapeutics [51,53,55,57,59,61,62,64−73] (Table 5). Pharmacopeial methods (Table S2) are available for the potency measurement of some protein therapeutics. Result of the bioassay can indicate quantity and HOS identity (bioidentity) of the protein.

Bioassays carried out for less complex protein therapeutics are not always suitable for mAbs whose molecules are more complex, e.g., several mAbs possess Fc effector functions in addition to a target binding-based mechanism by Fab [196,214,215]. Biological assay validation or verification is needed to demonstrate suitability of the potency test [80,81,89,90]. In general, bioassays are subject to a wider range of variations than that of physicochemical tests [31,186,214,215]. Balance between the measurement reliability for a quality control and the inherent variability due to the use of biological material in the test should be considered [214]. If such an uncertainty affects reliability of the potency test, physicochemical tests may be needed to give an extra-confirmation about HOS. To replace the potency assay with the chemistry-based tests, such methods should be capable of providing sufficient information about HOS with relevant correlation to biological activity [23,186].

*2.3.6. Additional tests for a drug substance (F)*

For concerns on chemical/physical instabilities, a pharmacopeial test on pH (Table S2) should be performed on DS which is stored as a solution. Appropriate, pharmacopeial tests with microbiological assays (Table S2), such as bacterial endotoxins test and total microbial count, should be performed on DS to determine microbiological contaminants [23,31]. As per ICH Q6B, “process-related contaminants” are any adventitiously introduced materials (e.g., chemical, biochemical, or microbial species) not intended to be part of the manufacturing process of DS or DP [23,97] (Fig. 2).

*2.3.7. Other general tests for a drug product (G)*

Other quality attributes of a parenteral DP that should be tested are pH and osmolarity of the formulation, owing to chemical/physical instability and patient tolerability concerns [31]. Pharmacopeial tests (Table S2) can be used for the measurement of these quality attributes [23].

*2.3.8. Additional tests for a unique dosage form/drug product (H)*

The additional tests include, but are not limited to, sterility, endotoxins, microbial limits, volume in container, particulate matters, uniformity of dosage units, moisture content, amount of a particular excipient (such as polysorbates), amount of a certain vaccine adjuvant, and uniformity of dosage units [23,31]. Pharmacopeial tests of these quality attributes (Table S2) can be referred to. Objectives of these tests are to control the dosage form dependent CQAs and to determine the chemical/biochemical/microbiological contaminants in DP. For parenteral products, the general chapter on their product quality tests should be considered and implemented accordingly [174].

### 2.4. Critical quality attributes of different types of protein therapeutics

Quality attributes of DS and DP listed above are common items in the specifications of protein therapeutics [23], regardless of different protein types (e.g., hormones, enzymes, mAbs, and Fc fusions). mAbs, and Fc fusions (Fig. S1), which are larger proteins with more heterogeneity, require more tests of product-related substances and impurities, particularly charge variants, glycosylation and other PTMs. Total protein concentration of heterogenous species may not reflect their quantity well, and the potency assay is needed for protein therapeutic quantitation. For a more complex molecule like a bispecific monoclonal antibody, quality attributes that can ensure dual specificity of the drug must be included in the specification. In addition to quality attributes of mAb, a chemical linker and a payload must be considered in setting the specification of ADCs (Fig. S1). Simultaneous characterization of identity, quantity, conjugated species and drug-to-antibody ratio of a lysine-conjugated ADC at its intact and subunit levels was demonstrated using MAM [216]. Some CQAs specific to ADCs are mentioned in Section 4, and in-depth discussions about analytical characterization of ADCs can be found in a review article [217]. In addition, elemental and mutagenic impurities and residual solvents which may associate with a synthesis of linker and payload are necessarily assessed [32−35,211]. Perspectives on testing CQAs which are universal in the DS and DP specifications of mAbs are summarized in Table 6.

## 3. Protein therapeutic specifications: acceptance criteria and statistics

According to ICH Q6B, the acceptance criteria of the specification are “numerical limits, ranges, or other suitable measures for acceptance of the results of analytical procedures which the drug substance or drug product or materials at other stages of their manufacture should meet” [23] (Fig. 1B). In establishing and justifying the acceptance criteria, all of these data should be considered: relevant drug development data (e.g., those from the detailed characterization, efficacy and toxicity studies), data from preclinical and clinical lots, data obtained from lots used for demonstration of manufacturing consistency, and data from stability studies [23]. Setting the acceptance criteria of specification can be considered in different ways.

### 3.1. Descriptive acceptance criteria

Acceptance criteria are justified and established based on a descriptive characteristic of the quality attribute. Descriptive acceptance criteria may be based on a comparison with a result obtained from a reference material, i.e., whether it conforms to the result from the reference material. Examples of the quality

**Table 6**
Perspectives on testing the (critical) quality attributes which are universal in the drug substance (DS) and drug product (DP) specifications of monoclonal antibodies (mAbs).

| Quality attribute | Perspective on testing |
| --- | --- |
| A. Appearance | • Physical state (powder or liquid), color, and clarity of solution can be visually inspected. |
| B. Identity | • As a qualitative analysis, the test should be highly specific to the protein therapeutic.<br>• Overall structure: Isoelectric point (pI) identity of an intact mAb can be obtained from an isoelectric focusing (IEF) technique. Unlike smaller and less complicated proteins, reversed phase liquid chromatography (RPLC)-UV is not used for the identification of an intact mAb due to the heterogeneity of mAb.<br>• Primary structure: RPLC-UV is used for the peptide mapping of mAb.<br>• Higher-order structure: Bioactivity outcome from a bioassay/potency assay are used to indicate the bioidentity of mAb. Physicochemical test can be used if it provides sufficient information about the structure with relevant correlation to biological activity. |
| C. Purity and impurities | • Analytical techniques should have enough specificity and sensitivity to indicate a small change, particularly for trace impurities. (Stability-indicating assay)<br>• Size variants: Size-exclusion chromatography is commonly used for the analysis of high molecular weight species while capillary electrophoresis-sodium dodecyl sulfate is suitable for the analysis of low molecular weight species.<br>• Charge variants: Ion-exchange chromatography and IEF electrophoresis are used in the analysis of charge variant isoforms and impurities (acidic and basic species)<br>• Post-translational modification (PTM) variants<br>- Intact protein level: RPLC, hydrophilic interaction chromatography (HILIC), and hydrophobic interaction chromatography can be used for the determination of related substances and impurities based on different hydrophobicity/hydrophilicity of different intact protein forms.<br>- Peptide level: Multi-attribute method (MAM) is an LC-MS-based peptide mapping method that can simultaneously determine several site-specific protein modifications in a single run.<br>- Glycan level: HILIC is often selected for the analysis of glycans/oligosaccharides released from mAbs. Derivatization of the released glycans is needed for fluorescence detection.<br>• Process-related impurities: Impurity profile depends on an expression system, excipients, equipment, and a container-closure system being used in manufacturing. Risk assessments are needed, and a test must be performed if there is a risk of having the impurity in DS/DP to evaluate whether the test should be included in the specification. |
| D. Quantity/protein content | • Analytical methods used in the quantitative analysis should be specific, sensitive, accurate, and precise enough for stability indicating.<br>• Quantity of mAb can be determined as the total protein concentration (using UV spectrophotometry), and a potency assay should be conducted to give information about the quantity of an active protein. |
| E. Potency/biological activity | • Quantitative assessment is conducted using a stability-indicating bioassay.<br>• Functional assay: Cell-based and some animal-based tests are used as functional assays to reflect a mechanism of action of the mAb.<br>• Binding assay: In cases where target binding is simply the mechanism of action, binding assay may be sufficient for the potency determination |
| F. to H.<br>Other general tests and additional tests | • Additional tests for DS: Pharmacopoeial tests on pH and microbiological tests should be performed.<br>• Other general tests for DP: Pharmacopoeial tests on pH and osmolarity should be performed.<br>• Additional tests for DP: Objectives of these tests are: (a) to control the dosage form-dependent CQAs and (b) to determine the chemical/biochemical/microbiological contaminants in DP.<br>• Pharmacopoeial requirements on injectable products should be considered and implemented. |
| Development trends: • Analytical quality by design (QbD) is a systematic approach for the analytical method development which utilizes analytical procedure performance understanding and control, based on science and quality risk management.<br>• Process analytical technology (PAT) and multivariate analytical procedures are developed to monitor product-related impurities, higher-order structure and some other CQAs for real time release testing. | |

attributes in this category are appearance, description, and identity (primary and higher-order structures).

### 3.2. Numerical acceptance criteria/limits

For certain tests, numerical acceptance criteria can be set relatively simply without the amount of data available (Sections 3.2.1 and 3.2.2). For certain quality attributes, with more difficulties, an appropriate data set with statistical analysis must be available and required to establish meaningful numerical acceptance criteria [44] (Section 3.2.3).

#### 3.2.1. Established regulatory or compendial limits

Limits are set based on the known impact to patient safety and are as part of harmonized pharmacopoeia [44]. Process-related impurities and contaminants are the quality attributes in this category. Examples are residual DNA [206,218–220], bacterial endotoxins [221], total microbial count [222,223], sterility [224], and subvisible particles [173,177]. In addition, compendial acceptance criteria for uniformity of dosage units [225], which has an impact to efficacy and safety, are fit with this group.

#### 3.2.2. Acceptance criteria based on experience with related products

Acceptance criteria are set based on relevant information available in the literature for a given class of protein therapeutics and a manufacturer's experience with that class of biomolecules. For early-phase specification of clinical lots, these numerical criteria are required to provide sufficient quality control of DS and DP. Later, more product-specific data are required to justify and establish the acceptance criteria with a better linkage to safety and efficacy, resulting in the wider/tighter acceptance criteria for late-stage specification [44]. Examples of the quality attributes in this group are protein concentration, potency, monomeric purity, and product-related impurities with an immunogenicity risk (such as HMW species and certain glycoforms) [44,97]. Many of the additional tests for DS and unique dosage forms (DP) and the other general tests for DP are also fit with this group, such as pH and osmolarity tests [44,97].

#### 3.2.3. Acceptance criteria to be established based on product- and/or process-specific data

For the quality attributes which are specific to the amino acid

sequence (i.e., protein specific) and/or process, proposed acceptance criteria may not be appropriate without sufficient data to support. Product-related substances and impurities, such as LMW species, charge variants, and PTMs, belong to this group [44]. Acceptance criteria for HCPs are also considered in this way [205]. Many manufacturers have used a "report results" criteria, with no specified acceptance criteria, for early-phase specification of clinical lots until more data are available to justify and establish the acceptance criteria [44]. Late-phase specification is then established based on clinical lots whose results in clinical studies ensure efficacy and safety of the protein therapeutic, after monitoring a measurement trend of the quality attribute, and after conducting a stability study with thorough considerations on related substances, impurities, and contaminants profiling. Those species that are uncommon and/or present at a significant level should be further studied to know their effect on efficacy and safety.

During the drug development, the knowledge obtained through the thorough characterization and specification tests of the protein therapeutic DS and DP at different development phases along with efficacy and safety information from preclinical and clinical studies provide a strong foundation for developing the late-phase specification. Examples of phase-appropriate specifications, with proposed acceptance criteria, for protein therapeutic DS and DP, can be found in the review articles [43,44]. A diagram of different phases in pharmaceutical development of DS and DP are shown in Fig. 3.

Control limits (lower control limit (LCL) and upper control limit (UCL)) should be set by a manufacturer to reflect and monitor variations and trends in a manufacturing process, including systematic and random errors of production and analytical processes and stability losses during storage and handling. After obtaining enough data from testing different lots of DS/DP, a standard deviation of a population ($\sigma$) can represent a random error or uncertainty. To obtain a target value which is an average value of a population ($\mu$), a systematic error should be assessed to account for a shift in the manufacturing process and analyte instability prior to testing. The control limits and acceptance criteria are designed with consideration on trade-off between process capability and product quality. Normally, LCL and UCL are warning levels for assessing manufacturing consistency, and process optimization is performed to improve consistency. These control limits are not aimed to be limits of the specification. On the other hand, acceptance criteria in the specification, which are limits at the release (lower release limit (LRL) and upper release limit (URL)) and at the end of shelf life (lower specification limit (LSL) and upper specification limit (USL)), are set to ensure the efficacy and safety of a product. Uncertainty of an analytical method should be considered when setting control limits and acceptance criteria to prevent a wrong interpretation of a test result due to underdetermination or overdetermination. Fig. 4 illustrates the control and specification limits. More information on how to set the acceptance criteria and statistical considerations can be found in these review articles [30,31,40–44]. This ordinary approach to set the acceptance criteria based on data obtained from pharmaceutical development, preclinical trials and/or clinical studies is called a "process experience-based" approach, while another approach which is "patient-centric" will be discussed in the next section.

## 4. Quality by design and patient-centric approaches in setting the specifications

### 4.1. Quality by design in biopharmaceutical development

Principles of quality by design (QbD) in pharmaceutical development and lifecycle management are included in the ICH Q8 to Q12 guidelines [36,78,226–228]. QbD requires process and product understandings which incorporate the science and quality risk management (QRM) [29,30]. Specification is one part of the overall control strategies of DS/DP CQAs [23]. In QbD approach, quality target product profile (QTPP), CQAs, critical process parameters (CPPs) and critical material attributes (CMAs) are defined and assessed based on their quality risks. CPPs and CMAs must be well controlled in the manufacturing to have acceptable test results of CQAs (Fig. 5A). Zhang et al. [145] demonstrated the use of product and process understandings from prior knowledge and initial characterizations to do quality risk assessment and determine CQAs for analytical similarity of their mAb biosimilar.

As mentioned in Section 1.2, the control strategy is based on a risk assessment [36] (Fig. 5A). For QRM tools which are well-accepted in the pharmaceutical industry, cause and effect diagram (Fishbone/Ishikawa diagram) is suggested for hazard identification, while failure mode effects analysis (FMEA) can be used for risk analysis and evaluation, i.e., ranking risks to DS/DP quality based on their severity, occurrence, and detectability. FMEA is also

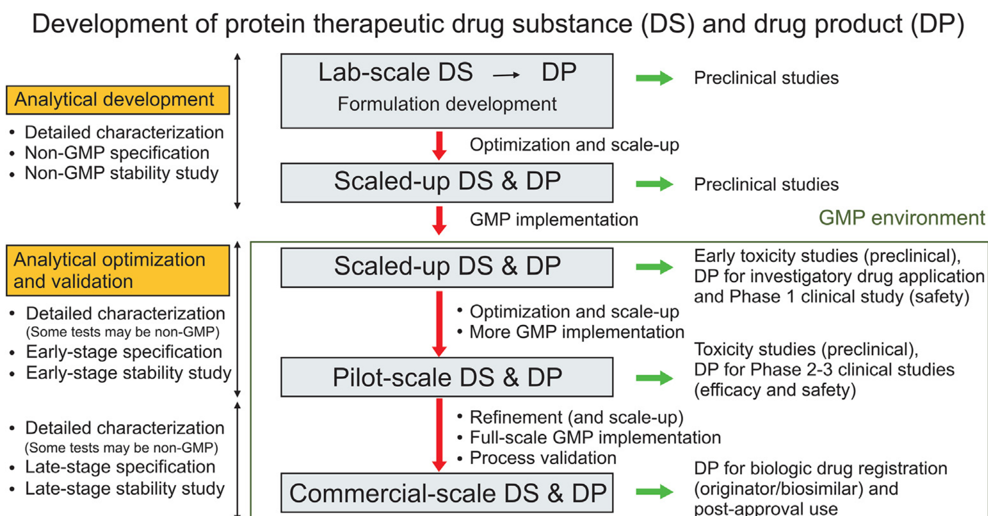


**Fig. 3.** Different phases in pharmaceutical development of the protein therapeutic drug substance and drug product. DP: drug product; DS: drug substance; GMP: good manufacturing practice.

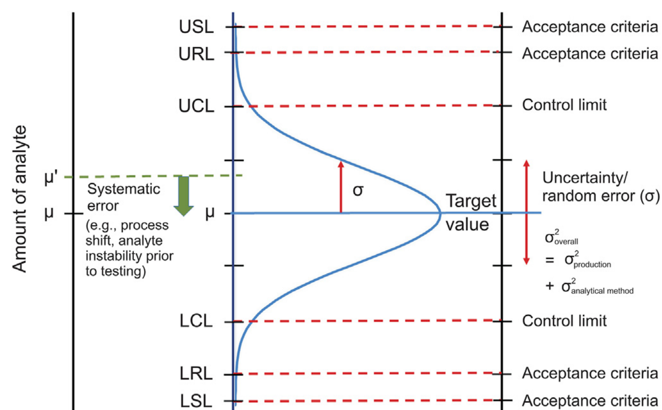


**Fig. 4.** Control limits of the manufacturing process and acceptance criteria (release limits and specification limits) of the specification. μ and μ′: population mean; σ, population standard deviation; $\sigma^2$: population variance; LCL: lower control limit; LRL: lower release limit; LSL: lower specification limit; UCL: upper control limit; URL: upper release limit; USL: upper specification limit.

suggested for evaluating the effectiveness of risk control strategies [36,229]. Control through careful material sourcing, equipment selection, process control, environment control, and/or another indicative release test are used for processes and attributes with lower risks, while those with higher risks, routine specification testing are also needed [32–35]. Some CQAs may not be controlled in the DS/DP specification if an in-process control or other control strategies are applied. Some of quality attributes which are not critical may be included in the detailed characterization, but not in the specification, to be informative and ensure the quality of biologics during drug development for its expected efficacy and safety. Extensive characterization, specification setting, and stability studies of the NISTmAb reference material 8671 under its life-cycle management and quality plan can be used as a case study of the QbD approach [230,231].

### 4.2. Patient-centric approaches

Even though QbD encourages a continuous improvement [29,30], it is stated in the ICH Q6B guideline that “Specifications should be based on data obtained for lots used in pre-clinical and clinical studies. The quality of the material made at commercial scale should be representative of the lots used in preclinical and clinical studies” [23]. In regular practices, a limited number of batches during pre-approval stages are used in setting the specifications, which raises a need for a revision of the guideline [38,39]. Development of the patient-centric specification (PCS) for DP was suggested by the International Society for Pharmaceutical Engineering (ISPE) PQLI® Patient Centric Specification Working Group to support patient-focused quality standards with the use of scientific and risk-based knowledge, as mentioned in the ICH Q8 and Q9 guidelines [36,78], and ultimately ensure the quality of DP [232,233]. Instead of setting the specification only based on the product variability over a limited number of clinical batches which may not reliably reflect the variability in a large population of the product, i.e., that of commercial manufacturing, PCS is established with a consideration on the actual effect of the product variability on product efficacy and patient safety. Importantly, the acceptance criteria should be set based on the available efficacy and safety data, e.g., data from *in vitro* and *in vivo* potency and toxicity studies of batches that represent the commercial manufacturing [31,232,233].

Strategies for setting PCS for biological products are comprehensively described in the review article by Ruesch et al. [31], with focuses and case studies on the acceptance criteria of purity, impurities, and potency tests and discussions on those of additional CQAs of ADCs such as potency, drug-to-antibody ratio, unconjugated mAbs (product-related impurities), free drug and its related impurities. Using risk-based assessment of the impact on efficacy and safety to patients along with rational selection of representative batches, proper statistical analysis of process capability, and stability considerations, the acceptance criteria of clinically relevant specification can be justified. With a caution against strictly limiting the manufacturing process to just two to three sigma (σ) which reduces the flexibility of manufacturing changes and continuous improvement which are suggested in QbD, a goal of PCS is to have tighter acceptance criteria on CQAs with the higher risk of impacting efficacy and safety, while allowing more flexibility for CQAs with the lower risk [31,232,233]. Development of pharmacopeial monographs and regulatory standards should have a consideration on PCS for the sustainability of standards [234].

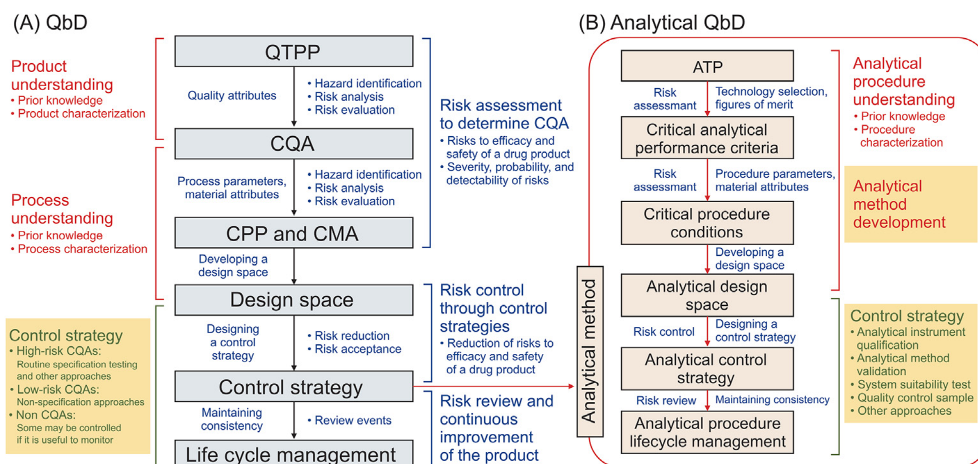


**Fig. 5.** Schematic diagrams of (A) quality by design (QbD) approach in the biopharmaceutical development in which specification testing can be considered as one of the control strategies, and (B) analytical QbD approach in the analytical method development in which analytical method validation is one of the control strategies. QbD requires process and product understandings which incorporate the science and quality risk management. ATP: analytical target profile; CMA: critical material attribute; CPP: critical process parameter; CQA: critical quality attribute; QTPP: quality target product profile.

## 5. Conclusion and trends in biopharmaceutical specifications

The quality of monoclonal antibodies and other protein therapeutics corresponds to their efficacy and safety to patients. To ensure good quality, there are many characteristics of DS and DP to be tested based on their quality risk. Specification is defined as a list of tests, references to test procedures, and appropriate acceptance criteria for the tests to set criteria which DS or DP should conform to. Quality attributes that should be covered in setting the specifications are appearance and description, identity, purity and impurities, quantity, and potency. Other general tests and additional tests are included for other quality attributes of DS/DP. Acceptance criteria are mainly set based on efficacy and safety profiles, with a suggestion to establish the patient-centric or clinically relevant specification. Scientific understanding and regulatory science perspectives of the protein therapeutic specifications are necessary to develop new analytical tools, standards, and approaches for the cost-effective quality control of biopharmaceuticals.

For regulatory compliance, considerations should also be given to the figures of merit of the analytical method, such as specificity, sensitivity, linearity, accuracy, and/or precision, which are associated with the acceptance criteria of the specification, and can be determined during analytical method validation/verification. Experimental procedures and results of performance characteristics of the methods should be submitted to a regulatory agency as part of CTD. In March 2022, a new ICH guideline on analytical method development (ICH Q14) [235] and a revised ICH guideline on method validation (ICH Q2) [82] were endorsed and are currently under public consultation. These guidelines suggest the use of science and risk-based approaches in the development, validation, and maintenance of the analytical procedures which are used as part of the control strategy for DS and DP manufacturing [82,235]. Enhanced approach for analytical procedure development and validation is based on QbD and lifecycle management to assure reliability of the analytical method, i.e., driving the analytical method lifecycle with the use of analytical target profile (ATP) and control strategy [82,235,236]. Analytical QbD is a systematic approach for the analytical method development which utilizes analytical procedure performance understanding and control, based on science and QRM. This approach begins with an objective that the result from the analytical procedure is reliable and fit for use. Quality risk assessment is performed to define analytical performance requirements (as ATP), select analytical technology, and design critical analytical performance criteria (as CQAs of the method) and critical procedure conditions (as CPPs and CMAs of the method). Analytical method development and ongoing controls, such as system suitability test and analysis of QC sample, are considered as control strategies to control a risk which has an impact on ATP. Analytical QbD is a life cycle approach which enables continuous improvement through change management when adopting new performance criteria, new technology or new procedure conditions [235,237,238] (Fig. 5B).

Development of multivariate analytical procedures and process analytical technology (PAT) for real time release testing is also included in the guidelines [82,235]. Reviews on the use of PAT, particularly IR and Raman spectroscopies, for in-process control of biopharmaceutical products from upstream and downstream bioprocesses are suggested to interested readers [239–242]. Raman spectroscopic methods were demonstrated to monitor product-related impurities, such as oxidized forms, aggregates, and fragments, higher-order structure and some other CQAs with a promising performance to be developed as PAT [243,244].

Another outlook is the development of analytical tools for investigating small conformationally-altered populations of protein therapeutics. Most of higher-order structural techniques and bioassays detect gross (weighted average) changes, so differences could be missed if conformer variants balance out with a larger number of native species [101,186]. Those product-related impurities may be immunogenic, and a control strategy should be established to determine their levels in DS/DP [245–248]. Techniques that have a potential to distinguish different conformers with sensitive detection of small populations are single-molecule fluorescence spectroscopy, immunological techniques against structural epitopes, native chromatographic and electrophoretic techniques with MS detection, HDX-MS and CL-MS at an intact or top-down level, native and collision-induced unfolding (CIU) ion mobility–mass spectrometry (IM-MS) [107,186]. However, utilizing such methods in the routine specification test requires costly instruments, and relevant correlation of physicochemical determination to biological activity should be established to avoid setting too strict acceptance criteria in the specification.

## CRediT author statement

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## Declaration of competing interest

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

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