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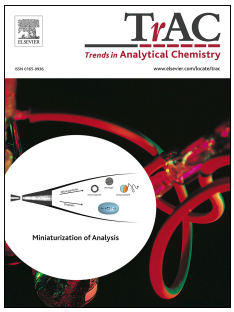

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Impurity Investigations by Phases of Drug and Product Development

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Abstract

Thorough knowledge and control of impurities is an expectation for the registration of pharmaceuticals. Actual and potential impurity investigations are phased during drug development to acquire the appropriate information necessary to ensure drug safety from the standpoint of patient exposure to impurities. Regulatory expectations and common practices for the timing of impurity investigations during development are discussed. Investigations for synthetic drug substances include process-related impurities such as intermediates, by-products, mutagenic impurities, residual solvents, and elemental impurities. Stress or forced degradation studies are used to investigate degradation impurities for both drug substances and products. The goals of stress studies conducted at different phases of development are discussed. Protein products have related considerations for impurity investigations, but the nature of impurities and technologies used for determining them can be quite different compared to classical synthetic molecules. Considerations for protein product impurities are discussed with an emphasis on process impurities in monoclonal antibodies.

Key words: drug impurities; drug development; process impurities; stress studies; forced degradation; monoclonal antibody impurities; monoclonal antibody purification

1. Introduction

Regulatory expectations for control of impurities in new drugs have been established through ICH guidelines for many years [1]. The Q3 guidelines outline requirements for the registration of new drugs and therefore represent the expectations for knowledge of impurity sources and controls that should be present as development is completed. Little guidance is given regarding expectations by phase of development other than acknowledgement that knowledge should increase and be applied to the manufacture and storage of drug substances and products. Regional guidelines supplement the ICH and sometimes offer more phase-related comments, but usually few specifics [2-4].

Drug development sponsors must determine the nature and depth of impurity investigations to conduct as the development process moves through clinical phases. Cost can be a major factor in the timing of these efforts. The high rate of attrition of new drug candidates entering clinical studies makes complete impurity investigations at early phases impractical. Patient safety is the primary consideration for impurities at all phases. All situations have specific considerations that depend on factors such as intended therapeutic use, dosage form, route of administration, duration of dosing, and patient population.

Impurity control is part of an overall control strategy developed for a drug product. Elements and development of a control strategy are described in ICH Q8, Pharmaceutical development, and related guidelines [5]. Impurities as they relate to safety are usually considered Critical Quality Attributes (CQA) of drug substances and products. It is also acknowledged in regulatory guidances that the control strategy develops over time as knowledge is gained [6].

This article will focus on the investigation of process-related impurities and degradation products for synthetic and bioproduct (specifically, monoclonal antibody) types of drugs. The investigation of impurities encompasses several interrelated topics such as identification of impurities, chemistry knowledge and analytical methodologies used for development and control, and setting specification acceptance limits for impurities. Decisions about the extent and timing of impurity investigations are sometimes company-dependent, so literature articles about specific company strategies are not plentiful. Therefore, the discussion represents the authors' experience and opinions in addition to publicly-available information. Regulatory-related references are provided when available.

2. Synthetic Drug Substances – Process-related impurities

2.1 Related Substance Impurities

A primary driver of impurity investigations throughout development is patient safety. In early clinical phases, not everything is known about impurities but materials used for pre-clinical

toxicological safety studies are often then used for initial human trials. In such cases, related-substance type impurities (i.e., compounds, either process-related or degradation-related, that are structurally related to the drug substance) are usually either controlled to levels at which the toxicological concern is minimal or are toxicologically qualified. The short duration of early clinical studies and close monitoring of subjects and patients also reduces the risk of safety problems caused by impurities. Specifications for impurities at early phases often reflect levels that have been observed in material used in toxicological safety studies [7]. With continued development and changes in the clinical exposures the specifications may change. Some firms choose to apply ICH identification and qualification thresholds at early phases. Teasdale et al. have recently proposed broader general limits for early phases with toxicological considerations based on total drug exposure to the patients [8]. An IQ Consortium working group proposed identification and qualification thresholds three-fold higher than ICH Q3 guidelines for related substances that could be applied through specifications or internal alert limits [9]. For registration and often at Phase 3, compliance with ICH limits is an expectation.

Starting materials, intermediates, reagents, catalysts and solvents used in the synthesis of a drug substance are obvious potential impurities in the drug substance [10]. Distance (i.e., number of steps) from the drug substance in the synthetic route is often related to the probability that a potential impurity will be removed prior to isolation of the drug substance. After the commercial synthetic route is chosen, impurity purging and fate studies are usually conducted to determine effective control points in the process. As development progresses, the structures of unknown impurities are identified and additional methods are developed, if necessary, to determine whether potential impurities are present or not.

Stereochemical control is expected at Phase 1 for single enantiomer drug substances. The timing of investigations of stereoisomers for compounds with multiple chiral centers will often be dependent on the complexity of the synthesis and how the chiral centers are introduced.

Impurities in starting materials are a regulatory concern and need to be controlled as part of the justification of establishing a regulatory starting material. Starting materials introduced close to the final steps carry a greater risk of introducing impurities in the drug substance, so the investigation and controls needed are usually more rigorous. The plans for impurity controls in starting materials are often the subject of discussions between FDA and the company at an end-of-phase 2 meeting. A recent ICH Q11 working group document addresses several issues, including impurity control, related to selection and justification of starting materials [11].

Analytical methodologies need to evolve as the overall impurity control strategy develops. Methods often progress from general screening conditions (typically reversed-phase HPLC with a broad polarity gradient) to methods optimized for impurities of interest at a given synthetic step [12]. Generic HPLC methods employing mobile phases compatible with mass spectrometric

detection are often used at early phases to facilitate impurity identification and are modified, as needed, for later-phase development. Phase-appropriate validation requirements for analytical methods have also been proposed [13, 14].

Considerations for the timing of specific types of other process impurity investigations are discussed below. Investigation of extractable and leachable impurities is described in another article in this issue.

2.2 Mutagenic impurities

ICH M7 provides guidelines for the assessment of impurities for mutagenic potential [15]. The guideline also gives limits for known mutagenic and potentially mutagenic impurities during clinical development. It is noted that for Phase 1 clinical trials of up to 14 days, only known carcinogens and mutagens need to be limited to acceptable levels as described in the guideline. Other impurities, even those with mutagenicity-alerting structures, can be treated as non-mutagenic impurities because of the short duration of exposure. The guideline acknowledges that not all impurities will have been structurally identified and assessed for mutagenicity at early stages. At registration however, a complete assessment of the mutagenic potential of impurities and control strategy for mutagenic impurities will need to be described. Typical approaches to mutagenic impurity control include attempting to remove them from the synthetic route, purging studies to show removal, sometimes with a higher acceptance limit at an intermediate, or establishing an M7-based acceptance limit at the drug substance. A more complete review of recent approaches for mutagenic impurity analysis and control are described in another article in this issue.

The need to control alkyl sulfonate esters is an example of a typical early phase regulatory expectation. Despite ongoing debate about the safety liabilities of these potential impurities or the lack of probability that they would be present [16], in the authors' experience, specification controls will be expected for these impurities, even at Phase 1.

2.3 Residual solvents

The solvents used in a synthesis are known and are usually specified and controlled at all phases. Standard methodologies, such as headspace gas chromatography, facilitate determination of most solvents used in drug syntheses at levels consistent with ICH Q3C. One approach is to determine levels of all solvents used in the process in the drug substance. Another approach is to control some solvents at earlier intermediates when they are not used downstream from that point. The approach taken can depend on complexity of the synthesis and number of solvents involved.

At later stages of development, residual solvent controls are usually needed for starting materials, especially those introduced closer to the end of the synthetic route. Certification that no class 1 solvents are used is also usually sought from the supplier.

The timing of investigations of impurities in solvents, such as benzene in toluene, may vary. Some firms may choose to perform such studies and institute controls at initial phases of development. Others may use a risk-based approach depending on the step in the synthesis where the solvent is used and controls on supplier quality. At registration, a control strategy will need to be in place for such impurities, whether that is by specification or by demonstration of adequate removal during the process.

2.4 Elemental Impurities

ICH Q3D has provided safety-based limits for elemental impurities in drug products and a risk assessment process for evaluating the potential for elemental impurities being present in the drug product. Controls for any metal-based catalysts used in the drug substance synthesis are needed from initial phases onward. Later in development, a risk assessment should be performed to evaluate other potential sources of elemental impurities, such as starting materials, excipients, manufacturing equipment, container/closure system, or water. Appropriate controls can be applied or data generated to support the risk assessment that specification controls are unnecessary. As with residual solvents, standard analytical methodologies are available that some firms use for specification control or data generation to justify that specifications are not needed [17]. Explicit controls for elemental impurities are generally considered to be unnecessary for biological products [18]. A risk assessment for the potential introduction of elemental impurities in individual biologicals is still expected, however. An FDA draft guidance includes the need to revisit elemental impurity risk assessments as part of change control for the product life cycle [19].

2.5 Manufacturing changes

As the drug substance synthetic route or process changes during early phases, there is the potential for new impurities. Different starting materials or intermediates are obvious candidates for investigation to determine whether existing analytical methods can detect them and whether they (or downstream analogs) carry through to the drug substance. Different solvents and reagents are also candidates for investigation as new impurities. The potential for the formation of different reaction by-products should also be examined during an impurity risk assessment for a process change. This could involve the prediction of potential new by-products, the potential for purging or carry-through, and the probability that the impurities method could detect them. The choice of a commercial synthetic route is a trigger for in-depth investigations of impurities, especially if clinical development is likely to advance to phase 3.

Any post-approval changes to drug substance manufacturing should be evaluated for the potential impact on impurity profile. This includes a wide range of possible changes in addition to changes in route or materials used. For example, changes in manufacturing site, process set points, scale of manufacture, and sources of purchased materials should include an evaluation of impact on impurities. An interesting example of a seemingly benign change was described by Reddy et al. who found a new impurity in repaglinide after the supplier of the dicyclohexylcarbodiimide (DCC) coupling reagent used in the process was changed [20]. Cyclohexylamine present as an impurity in DCC from the new supplier gave rise to a new impurity in the drug substance. This highlights the need for use-test evaluations of new suppliers in addition to checking conformance to existing specifications.

3. Degradation products in synthetic drug substances and drug products

Stress testing is the main tool used to predict and develop an understanding of the stability of a particular drug substance and drug product. Stress testing goals include investigating the likely and actual degradation products that can be formed along with developing analytical methodology(-ies) to separate, detect, and quantify degradation products. In the last several years, several key publications have discussed various aspects of stress testing in detail, and the reader is referred to these for a more thorough discussion [21-25].

As a new drug entity progresses from discovery to preclinical to clinical stages of development and eventually to the market, knowledge about its stability (and the degradation pathways and products) is expected to increase. Thus, stress testing is typically not a “one time” event but rather something that is carried out at different stages of the “life cycle” of a drug substance and drug product, with different goals, strategies, and level of thoroughness [26]. This is especially true for the development of novel drugs where the attrition rate is typically very high (e.g., 90% or even higher); it is not cost-effective to perform the level of research needed for a marketed product for every new drug candidate. The primary goals are to ensure efficacy and safety for the patient (throughout the clinical trials or ultimately the marketed shelf life). The shelf life of most drugs is limited not by efficacy (i.e., not by the level of the parent drug), but rather by safety (i.e., by the formation of degradation products at levels of concern).

3.1 Drug Discovery Stage

The goal of stress testing or stability studies at this stage is primarily to determine whether or not a compound has stability sufficient for the desired routes of administration during clinical studies. Such studies are typically short in duration, limited in scope, and use analytical methodologies that are typically generic (i.e., with an emphasis on high throughput, not specifically designed for the individual compound). Degradation products are typically viewed as “peaks in a chromatogram”, not as identified degradants. It may be prudent to evaluate the theoretical potential for formation of mutagenic degradation products for particular

structures/scaffolds, since controlling degradation to the low levels required for mutagenic degradants may be very difficult, and could potentially threaten the developability of the drug [27]. Over the last 10 years, the software program Zeneth has developed into the most sophisticated tool available for *in silico* predictions of theoretical degradation pathways [28, 29]. It is also useful at this stage to access the knowledge gained from previous studies on compounds with similar structures, from either published or company internal information.

Since early batches of drug substances are typically not representative of the solid form(s) (e.g., polymorphic, salt, free base/free acid, or co-crystal form) that will be used in the clinic or on the market, solid state stress studies may not accurately reflect potential stability issues of the clinical or final marketed form.

3.2 Preclinical to Phases 1/2

While the reporting of stress testing studies is encouraged (but not specifically required) in Phase 1 or 2 studies [2, 3] they are expected to be carried out on the drug substance with a focus on ensuring that stability can be maintained throughout the clinical trial; stability-indicating analytical methods that are specifically developed for the drug substance are expected [26]. No mention is made of stress testing of the drug product. In the early stages of development, the focus of method development is more on selectivity and less on robustness [30]. In some cases, highly resolving generic methods have also been applied at this stage, which may provide the needed selectivity for a variety of compounds [31]. Generally, identification of degradation products observed during stress testing is not critical during this stage, although there are many times when such information can be very useful to the further development of the compound; typically, structural information at this stage is limited to data obtained through LC/MS analyses (e.g., molecular weight, fragmentation, etc.) [26].

3.3 Phase 3 to NDA Regulatory Submission

Stress testing studies, with a full understanding of the "inherent stability of the drug substance, potential degradation pathways, and the capability and suitability of the proposed analytical procedures" are expected to be completed by or during Phase 3, and certainly for the marketing application. The goals of stress testing at this stage are to understand all potential stability issues related to degradation product formation including storage, distribution, short-term temperature excursions, formulation, and even potential patient "in-use" stability issues, as well as to provide a thorough foundation for validation of stability-indicating analytical methods for the marketed life of the compound. A complete understanding of potential degradation products and pathways (including mass balance understanding) should be developed, with a perspective that this information will form "an integral part of the information provided to regulatory authorities" in the marketing authorization submission. ICH

Q3A and Q3B reporting, identification, and qualification thresholds are typically fully applied at this stage of development for formal stability studies.

It is worth noting here that any degradation products for which structures (potential or actual) have been elucidated should be assessed for mutagenic potential, per the ICH M7 guidance on mutagenic impurities [15]. Several researchers have published articles to help companies navigate the degradation product implications of ICH M7 [32-34].

3.4 Line Extensions (New formulations, new dosage forms, new dosage strengths, etc.), Currently Marketed Products, and Generics

After registration, changes to the drug substance or drug product manufacturing process are often desired for cost reduction, quality or reliability increases, or environmental impact reduction. Manufacturing site and scale changes are also common. Risk-based guidances, such as ICH Q9, can aid in assessing the significance of a process or formulation change which may require stability studies to be conducted to demonstrate that the proposed changes do not adversely impact the already established stability characteristics (e.g., degradation rate or profile) of the product. A rapid stability assessment, i.e., one that requires a much shorter time than typical accelerated or long-term studies, is desired. A rapid stability assessment is also desired for line-extensions involving new formulations or different strengths of an existing product. Olsen et al. have described the use of “highly accelerated” conditions for comparative stability studies or for developing stability models useful for a broad range of conditions [35]. In this mode, elevated temperatures and/or humidities beyond the ICH accelerated stability conditions are used to compare the stabilities of products made in different ways or to develop predictive models. Such highly accelerated or stress studies can be useful in evaluating process changes where a baseline of knowledge about the degradation pathways and rates of degradation of the compound already exists. Information about the stability of new formulations of existing active components can also be obtained quickly using highly accelerated conditions. Waterman has developed an approach using a humidity-corrected Arrhenius equation with elevated temperatures to develop product-specific models that can be used for accurate chemical stability and shelf-life predictions, usually from data collected over a 2-week period [36]. Such accelerated studies may reveal stability issues much more rapidly than traditional methods and lead to more efficient and effective drug development.

Another important consideration during the lifecycle of a drug is the development of new dosage strengths, new dosage forms, new formulations, and alternate routes of administration. Each new development will require new or modified stress testing and/or accelerated stability studies, as it cannot be assumed that degradation rates and pathways will remain the same as those in the original product. New or modified analytical methodologies may also be required, and therefore, new or revised accelerated stability studies will need to be performed as part of the stability-indicating method development process. New or modified analytical

methodologies can also lead to the discovery of new impurities (in line-extensions and even in existing products) that were not detected with previous methods.

At the time of patent expiry, publicly available data on stress degradation studies is often limited, that is, either not published or held as proprietary by regulatory authorities. Additionally, the compendia (e.g., USP, PhEur or JP) often do not have monograph methods established, and if they do, even if such methods are purported to be stability-indicating, the information in the established method may not be sufficient to discern this. Therefore, non-innovator companies will likely need to conduct their own set of stress/accelerated stability studies to (a) establish a thorough understanding of potential degradation products for the drug substance and drug product, (b) demonstrate for the new source of drug substance or drug product that the synthetic pathway or process (for drug substance) and formulation and process (for the drug product) can be adequately characterized with appropriate test methods, and (c) guide the development and scale-up for the drug substance and drug product manufacture.

4. Impurities in Protein Therapeutics

Traditional small-molecule pharmaceuticals and precursor intermediates usually undergo purification by isolation as crystalline solids during the synthesis. The manufacturing steps introduce impurities that need to be carefully assessed and removed during these purification steps. In contrast to small-molecule drug substances, protein therapeutics are made by living cells. With the advent of recombinant DNA technologies, it is now possible to engineer and express various proteins in bacterial (e.g. E. coli) or mammalian cell lines (e.g. Chinese hamster ovary, CHO cells). While the therapeutic proteins of interest are produced in larger quantities, the cells also co-produce other biologics (proteins, DNA, etc.) that are considered as impurities. Host cell proteins (HCPs) are encoded by the organisms and unrelated to the intended recombinant product and must be removed during downstream purification since these could potentially induce immunogenic responses in patients.

Monoclonal antibodies (mAbs) are a significant portion of marketed biologics in the US and Europe with over 64 products approved and more than 200 molecules in clinical development. Many biotechnology companies are focused on different forms of antibodies or antibody fragments for clinical development and have embarked on a platform approach for purification to get to clinical studies as fast as possible. Most mAbs are produced in mammalian cell lines, like CHO cells, and are typically purified using a combination of a Protein A affinity step followed by two or three polishing steps. Each of these steps is useful in removing certain types of impurities from the cell culture mixture and will be the topic of discussion in the next few sections. Monoclonal antibodies undergo chemical and physical changes during production, processing and storage. Chemical modifications such as isomerization/deamidation or oxidation may lead to changes in the charge profile of the mAb and are typically not considered process

related impurities. Product impurities including chemical modifications or high molecular weight species (e.g. aggregates) are somewhat expected for liquid drug products. However, there is an expectation that a thorough risk analysis and extended characterization study be performed to understand the various degradation pathways for the protein during normal processing and storage in line with the ICH Q6B guideline [37]. Similarly, post-translational modifications that arise during cellular expression including modifications such as glycosylation or disulfide bond isoforms are not necessarily considered product or process related impurities, but need to be thoroughly characterized. This review deals mainly with risk assessment and characterization studies that are performed or necessary for impurities that are co-purified during mAb production. The reader is referred to a critical review of *in vivo* and *in vitro* mAb modifications and characterization by Liu et al. [38] and an article in this issue on trends in research on impurities in biopharmaceuticals.

4.1 Typical purification steps for monoclonal antibodies and their associated clearance capabilities

Protein A chromatography is typically used as the first step in an antibody purification process due to its capacity for extensive removal of process-related impurities such as HCPs, nucleic acids, cell culture media components and various virus particles. Protein A has several Ig-binding domains and binds to the Fc region of several IgG formats with high affinity (in the order of 108 M^{-1}). This property is of significant value during purification of the IgG therapeutic from harvest cell culture fluid (HCCF) and is routinely used for affinity purification of the antibodies. A histidine residue on protein A (His137) is known to interact with another histidine residue on the IgG antibody (His435) through electrostatic interactions. The protein A bound antibody is eluted at low pH wherein both the histidines are positively charged resulting in electrostatic repulsions.

Strong attractions between the HCPs and the therapeutic IgG are possible that could potentially make it difficult to purify during a protein A purification step. Levy et al. have recently shown that product fractions of protein A affinity purifications contain more HCP than those fractions without the mAb [39]. Another possible pathway to introduce HCPs into the final pool is when the HCP species bind to either the chromatographic ligand or the resin backbone (e.g. protein A in this case). In either case, some amounts of impurities typically are retained in the protein A pool and further purification is deemed necessary. Since the protein A resin is recycled over 200 times, it is imperative to understand its impact on the performance of the protein A purification step. Carter-Franklin et al. have shown that intact Protein A leaches into the purified antibody or the HCCF [40]. This and other impurities necessitate the use of other chromatographic steps for further purification.

Most companies use IEX as a polishing step in antibody purification wherein it is ideal for reducing high molecular weight aggregates, charge-variants, residual DNA, some host cell

proteins, leached Protein A and any remaining viral particles. Specifically, anion exchange (AEX) chromatography uses a weakly basic or positively charged resin (e.g., diethylaminoethyl cellulose (DEAE)) to remove HCPs, DNA, endotoxin and leached Protein A. Additionally AEX can also help with product-related impurities such as dimer/aggregate, endogenous retrovirus and adventitious viruses. Cation exchange (CEX) chromatography utilizes either strong (e.g. sulfopropyl) or weakly acidic (e.g. carboxylic) groups on a resin to purify the antibody pool. While process-related impurities such as DNA, some host cell protein, leached Protein A and endotoxin are removed in the load and wash fraction, CEX specifically helps in purifying antibody by products such as deamidated products, oxidized species, N-terminal truncated forms, and high molecular weight species.

Complementary techniques such as hydrophobic interaction chromatography (HIC) can also be used in addition to Protein A and IEX methods to further separate proteins and impurities based on their hydrophobicity. HIC in flow-through mode is efficient in removing a large percentage of aggregates with a relatively high yield while in a bind-and-elute mode it is used to remove process-related and product-related impurities from the antibody product. The majority of HCPs, DNA and aggregates can be removed from the antibody product through selection of a suitable salt concentration in the elution buffer or use of a gradient elution method.

4.2 Impurity characterization

Resins containing Staphylococcal Protein A are typically used during purification of mAbs during process development. It is possible that trace levels of Protein A leach into the final formulated drug substance. Many companies use an ELISA that utilizes anti-protein A antibodies for detection and quantitation [41]. These studies are typically done prior to any clinical use and typically even prior the Phase 1 studies. Since there is a possibility that the formulation components may interfere with the ELISA format, optimization for leached Protein A removal is done on a continuous basis throughout the program. Similarly, host cell DNA could potentially contaminate the purified drug substance. Several analytical methods have been qualified for use to help detect trace amounts of host cell DNA. Most commonly used are the Pico green assay, hybridization assays, qPCR or rtPCR and threshold assays. Amongst the tested assays, the inter and intra-lab assay variability for the qPCR was much lower [42].

Similar to any immunogenicity risks from Protein A and host cell DNA , source materials and adventitious viruses introduced during protein production present viral contamination risks. Source materials can include human plasma, cell lines, and human/animal tissue. The risk of viral contamination is higher for human- and animal-derived source materials than for non-biological materials and therefore viral inactivation processes are very important during development. Low pH (typically pH < 3.6) has been shown to inactivate enveloped viruses. Robust process development including validating hold times for viral inactivation is a

mandatory step during process development. Processes that include virus-reduction filters typically remove non-enveloped viruses. Many chromatographic steps including IEX provide two to three logs of virus removal and many manufacturers use qualified or validated steps early on in process development in order to de-risk viral contaminations from biotechnology products.

In addition to host cell DNA, leached protein A or virus particles, the protein drug substance could potentially have other impurities such as host cell proteins. Most companies utilize an ELISA method to characterize HCPs throughout all phases of development. In the initial phases of development (preclinical tox studies to Phase 1 or Phase 2), the biotechnology industry typically uses commercially available ELISA kits. Some companies may also utilize specialized or customized ELISA kits depending on the specific organisms or cell culture systems they use to produce most of their antibody products [43, 44]. While commercial kits may have significant advantages in terms of resources and development, more customized assays may be necessary as the program proceeds from early to late development and into the commercial realm. A platform-based approach may be suitable if the company uses the same expression system for producing a variety of therapeutic candidates since the proteome and the HCPs would likely be similar.

While not considered as a part of process impurities as discussed above, chemical and physical modifications of mAbs may occur during production, processing or long-term storage that are considered as product-related impurities. Chemical and physical degradation pathways are considered as a part of the product microheterogeneity and a thorough analytical characterization in line with ICH Q6B guidelines is expected. Typically charge changes via deamidation are analyzed using ion-exchange chromatography or imaged capillary isoelectric focusing (iCIEF) or mass spectroscopic methods. Physical degradation pathways, including formation of high molecular weight species (or aggregates) are typically characterized by size exclusion chromatography, though orthogonal methods such as analytical ultracentrifugation (AUC) are also recommended. While product stability may limit shelf life, heterogeneity in the mixture may impact pharmacokinetics (PK) or cause immunogenicity risks. Khawli et al. have shown that mAb charge heterogeneity generated during routine manufacturing had minimal effect on various biological assays, such as FcRn binding, potency or PK properties of an IgG1 in healthy rats [45]. While immunogenicity of protein aggregates and subvisible particles has been an active area of research, recent data suggests that only subvisible particles that have extensive chemical modifications within the primary amino acid structure could break immune tolerance in the human IgG1 transgenic mouse model [46]. A thorough risk assessment and characterization of aggregates, subvisible particles and immunogenicity risks associated with them is out of scope for this review and the reader is directed to other articles [47, 48]. Risk based approaches for process-related impurities are described below.

4.3 Risk-based approaches for process-related impurities

While ELISAs are efficient methods for assaying holistic information about the HCP population, characterization of specific HCPs cannot be made by ELISA alone. Characterization of specific HCP species and demonstration of suitability of the ELISA for a given process and product must therefore employ orthogonal techniques such as western blots and/or proteomic tools such as 2D gel electrophoresis and mass spectrometric analysis of the impurities. A product specific HCP ELISA or orthogonal method is more resource intensive and may be expensive if applied for each product early on, especially since many candidates will fail early on in development. Given this situation, it makes more sense to spend time and resources during later stages of development (e.g. Phase 3 and/or commercial scale).

One needs to consider that polyclonal antibodies used in the ELISA kit depend on the antibody serum developed against HCPs and may not represent all the HCPs equally in an ELISA response. A response indicates that the HCP components are equally weighted and similarly, a negative result indicates that no HCP in the mixture could potentially cause immunogenic effects. Overall, this is the limitation of using ELISA kits and sensitivity of the assay, its degree of coverage of the HCP, and risk-based approaches are needed. A risk-based approach needs to have a strong scientific basis to estimate and understand the impact of types and concentrations of HCPs that will not have adverse impact on the product quality of the therapeutic. Wang et al. have recently reported a risk-based approach for HCPs in biological products [49]. Champion et al. also reported recently that most HCP impurities in FDA approved products are < 100 ppm [50]. This level of impurity has turned out to act as a guidance to the biotechnology industry to set HCP levels in their products, though this value does not take into account specific considerations around different HCP species, patient population, or dosing regimens. Therefore, acceptable levels of HCPs in a given product are typically approved on a case-by-case basis by the health authorities. The ultimate suitability and acceptability of the HCP test methods are based on the results that the sponsor companies obtain both in detecting and quantifying the residual HCP levels in registration batches that are usually made at the commercial scale. It is rather difficult to fully understand the immunogenic impact of individual HCPs in a particular patient population. Using a variety of *in vitro* and *in silico* tools Jawa et al. have recently reported that HCPs typically found in biotechnology products and that would follow ICH Q6B [37] have low to no impact on immunogenicity [51]. While potentially good news for various biological products produced using platform purification processes, this also necessitates continuous improvement to understand HCPs. Novel orthogonal methods to accurately estimate and determine HCPs and understand their potential impact to patient safety are needed. To this end the use of LC-MS has been shown recently to be the workhorse for HCP identification [52, 53], though the use of other *in silico* analysis is also growing [54].

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Highlights

- Impurity investigations increase in scope and depth as development progresses
- Common practices for impurity investigations by phase of development are described
- Stress study depth and goals by development phase are described
- Purification and determination of process impurities in mAbs are described
- Considerations for determination of host cell proteins in mAbs during development are discussed