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Review

Host cell contaminant protein assay development for recombinant biopharmaceuticals

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Abstract

The efficiency and consistency of a biopharmaceutical purification process determines drug quality, including which specific types and concentrations of residual host cell or process contaminants may remain. Commercial reagents and generic analytical methods are available for quantitating most of these contaminants. However, no generic assay is available for quantitation of the specific contaminant host cell proteins (HCPs) which are unique to a novel purification process. Because of this, proprietary reagents and assays must be developed for the quantitation of process-specific HCPs in each biopharmaceutical drug. The need to develop proprietary reagents which are both sensitive to, and specific for, potentially complex mixtures of unique contaminant proteins has defined what is acceptable methodology for development of quantitative HCP assays. Within the biopharmaceutical industry this need is most often satisfied by the development of multi-analyte HCP immunoassays based upon the null cell mock purification model. Confidence in the quantitative nature of a given HCP assay, and the validity of analytical measurement obtained by the assay, is dependent upon empirical demonstration of the unique stoichiometry of the HCP assay reagents. In conjunction with other analytical and validation methods, an HCP immunoassay may be thought of as a necessary quantitative tool for the optimization and validation of biopharmaceutical purification process efficiency and consistency, rather than as an end in itself.

Contents

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1. Introduction

Recombinant biopharmaceuticals are treated like biologicals with respect to quality assurance for purity [1,2], in that the efficiency and consistency of the purification process determines the quality of the product. The design and operation of a given process [3], therefore, will define the specific types, and amounts, of residual host cell or process contaminants that will remain in the bulk drug. These potential bioprocess contaminants may include items as diverse as: cell culture medium serum proteins; immunoglobulin affinity ligands; Protein A or Protein G affinity ligands; viruses; endotoxin; DNA; non-protein cell wall constituents; and host cell proteins (HCPs). Removal of each of these process and host cell contaminants to acceptably safe levels must be assured both by development of specific contaminant assays and by rigorous process validation [4–23]. In a broad sense, minimization of all forms of contamination in a therapeutic biopharmaceutical is a matter not only of safety, but of pharmaceutical elegance.

Commercial reagents and standardized analytical methods are available for detecting and quantitating most contaminants including: cell culture media proteins (H. Merrick, personal communication) [24]; immunoglobulin [25]; affinity ligand proteins [25]; non-protein cell wall constituents [26]; endotoxin [27]; DNA [28–31]; or viruses [32]. In contrast, in the presence of a vast excess of recombinant protein product, no generic protein assay for quantitation of undefined, complex mixtures of process-specific HCPs is feasible [4,10,13,33]. Because of this, proprietary reagents and assays must be developed and validated for the quantitation of process-specific HCPs. The evolution of this type of HCPs analytical development has been predominantly industry driven, because it has been essential for the development, optimization and validation of proprietary biopharmaceutical purification processes. These coordinated activities are a natural extrapolation of total quality principles [16,18]. Because of their proprietary nature, publication of relatively detailed descriptions of very recent developments has often been significantly delayed.

2. HCP assay development is specification driven

In the final analysis, process design, process optimization, process validation, and contaminant assay development, are specification driven. Thus, it is imperative to identify the approximate specification range expected for HCPs (or any other contaminant), based upon the prospective use of the product [3], as well as current analytical capability. Ultimately, acceptance of a given HCP specification (or the absence of a specification) by regulatory authorities is determined on a "case-by-case" basis [7,9,20], and depends upon a scientifically sound, multifaceted, quality assurance rationale [2,4,5, 10,11,14].

Given the current regulatory environment, and the current state of analytical capability, it is always prudent to approach contaminant specifications conservatively. Thus, the acceptability of an HCP specification for any given biopharmaceutical will vary depending upon combinations of a variety of mitigating factors including: dose size; acute versus chronic administration; use as an analytical or diagnostic reagent, rather than as a therapeutic drug; use as a vaccine; the nature of the expression organism (e.g. prokaryotic versus eukaryotic, untransformed versus transformed cells); and any unique "risk versus benefit" considerations [1–15,20–22,40].

For example, a therapeutic immunoglobulin G (IgG) expressed by a non-recombinant murine hybridoma cell line may require no specification assay for HCPs, although analytical and process validation assurance of the removal of typical cell culture medium proteins like BSA, bovine IgG, insulin, or transferrin is certainly expected (A. Lawton, personal communication). This rationale is based upon many years of manufacturing experience using stringently validated, standardized, immunoglobulin purification methods.

In contrast, a recombinant biopharmaceutical with a novel purification process will require HCP assay development. The required level of quality assurance, and the stringency of the specification, will depend upon one or more of

the factors cited above. A recombinant vaccine which is expressed in yeast, and administered in only one or two doses, may have a relatively high HCP content, e.g. 0.22% [1,34]. In contrast, a recombinant growth hormone produced in bacteria, and destined for chronic administration, will require a much lower HCP content, e.g. less than 10 parts-per-million (ppm) [4,35].

During the earliest stages of purification process design and evolution (pre-IND and into early Phase I [19]) it is advisable to meet informally with regulatory authorities to address the issue of acceptable specification ranges for a novel biopharmaceutical, with special emphasis upon the nature of the purification process, and the intended clinical use of the product.

3. The consensus HCP analytical triad

A consensus quality assurance analytical "triad" has evolved for the control of HCPs in recombinant therapeutic biopharmaceuticals. This strategy includes: sensitive silver staining (and immunoblotting) of electrophoretic gels; rigorous process validation clearance studies; and the development of quantitative, process-specific HCP assays [4,5,10,11,13,16–18,36–40]. The electrophoresis-based characterization methods [40–43], and clearance studies [17], are methodologically generic. In contrast, the specific methodological requirements for development of quantitative HCP assays have been constrained by the need to develop proprietary reagents which are both sensitive to, and specific for, potentially complex mixtures of unique contaminant proteins at very low concentrations [4,44]. In this sense, the quantitative nature of the multi-analyte HCP assay, and the validity of analytical measurement obtained by it, is dependent upon empirical demonstration of the unique stoichiometry of the HCP assay reagents.

4. The requirement for sensitivity, specificity, and stoichiometry

Demonstration of assay sensitivity is not arbitrarily a matter of mere analytical capability [45]. There is a practical requirement to quantitate residual HCPs at a level which is "suitable to obtain regulatory approval". There is no a priori presumption that residual HCPs from one expression organism are more likely to be deleterious to the recipient than are HCPs from another expression organism. However, the intent has always been to avoid "unsafe" levels of residual HCPs which might lead to toxic or immunological sequelae [44], and this concern is not entirely trivial. During early clinical administration of recombinant human growth hormone, it had been observed [46,47] that unacceptable levels of residual *Escherichia coli* (*E. coli*) HCPs may not only elicit anti-HCP antibody, but may also induce an "adjuvant effect" which results in elicitation of undesirable antibody against the biopharmaceutical protein itself [48]. This problem was ameliorated by improved purification methods which significantly decreased the bacterial HCP content of the hormone. No specific anti-HCP antibody elicitation was observed in recipients of recombinant human growth hormone of mammalian cell origin [49].

What is an acceptably safe level for residual HCPs? A detection range of 1–100 ppm of residual HCPs has been quoted as a regulatory (and analytical) benchmark for therapeutic proteins [20]. However, as will be seen later, leading biopharmaceutical firms have designed processes which consistently limit residual HCPs to a range of 1 to 10 ppm, and correspondingly have implemented assays which will consistently quantitate HCPs within, or below, that range [10]. Therefore, domestically, and internationally, for either human or veterinary pharmaceuticals, a useful working target for HCP content is 10 ppm [40]. The sensitivity and specificity of any unique HCP assay which is used to support such a target should be demonstrated accordingly.

Specificity must be defined by the empirical assignment of proprietary HCP detection reagent stoichiometry. These detection reagents are most often polyclonal IgG preparations which have been elicited against a process-specific immunogen that represents the "most probable" [44,52] contaminant HCP which consistently result from a unique purification process.

Thus, combined analytical sensitivity and

specificity has been achieved by employing these proprietary, process-specific HCP immunogens, and their corresponding proprietary, polyclonal, anti-HCP immunoreagents, in the development of "multi-analyte" (multi-antigen) immunoassays [50,51]. These methods generally employ a "sandwich" format [52] and include, but are not limited to: radioimmunoassay (RIA); immunoradiometric assay (IRMA); enzyme-linked immunosorbent assay (ELISA); antigen selected ELISA (ASIA); immunoligand assay (ILA); and electrophoretic, or dot, immunoblots.

5. HCP immunoassay rationale

A quantitative, process-specific, HCP assay may initially be intended for use as a highly sensitive specification assay for profile of the bulk drug. However, it is essential to recognize that HCP assay development is not mutually exclusive of the other parts of the HCP quality assurance analytical triad. By judicious timing of purification process development [76], the anti-HCP immunoreagents which are elicited for use in the specification HCP assay can have a positive, iterative, impact upon optimization of an evolving purification process [77]. This might include qualitative probing for HCPs in electrophoretic immunoblots, or quantitation of HCPs in purification process aliquots. In the later stages of process development HCP assays can ultimately be employed to support definitive process validation studies at full manufacturing scale [76,77]. In this way, a process-specific HCP assay is a means for validating that a process is consistently able to meet an acceptable HCP specification. If a process is designed appropriately for specific removal of contaminants [78] and validation is done properly, it may adequately demonstrate that a specification assay per se will not be required. However, timing is critical. Prolonged timeframes must be anticipated when planning for the manufacture and characterization of complex HCP immunogens, the elicitation, purification and qualification of anti-HCP immunoreagents, and the development and validation of multi-analyte HCP immunoassays. In this sense it is advisible to plan HCP immunoassay development as if it were required for a specification assay.

6. Process-specific HCP immunogen preparation

Within the biopharmaceutical industry the generally accepted paradigm for quantitative, process-specific, HCP assay development is the null cell mock purification model [4,10,13,16, 33,44,51–53]. A null cell (or blank cell) is a version of the expression strain that cannot express product. For example, for a prokaryotic expression system, a bacterial null cell is the expression host cell strain which contains a plasmid vector that has been cured of the expression gene.

For some eukaryotic expression systems the null cell may be more difficult to define. For instance, an acceptable null cell for a recombinant myeloma which expresses IgG as a product might be a "non-secretor null parental cell" (M.A.C. Costello, personal communication). In any event, the genotypic and phenotypic relationship of the null cell strain to the expression strain, and the analytical rationale for specifically selecting it, must be clearly defined and documented as early as possible during purification process development.

Mock fermentation of the null cell strain, and purification of null cell HCPs, should be executed under normal production conditions, and "acceptably close" to normal production scale. Regulatory input may be required in order to determine what production level is acceptable, in that full scale HCP manufacture for some eukaryotic expression systems may be impracticable, or possibly prohibitively expensive (V.R. Anicetti, personal communication). Ideally, subsequent isolation and purification of the null cell HCP should proceed to that point in the purification process at which the biopharmaceutical would normally approach 95–99% purity. This potentially complex mixture of null cell HCPs conservatively represents the "most probable" [44,52] downstream HCP contaminants, and is used as an immunogen for eliciting proprietary,

process-specific, HCP immunoreagents. A substantial amount of this preparation (e.g. 200 mg or more) may be required, not only for specific use as an immunogen, but for other purposes such as analytical characterization, possible use as an immunoaffinity matrix [58], and use as an assay standard over the lifetime of the product. Acquisition of this immunogen is usually time-consuming, labor intensive, and expensive.

After this immunogen is isolated, the purification process steps upstream of the immunogen selection point, and the expression organism itself, should not be significantly altered. To do so may invalidate all previously completed HCP assay development activities. After assay development is initiated, any potential changes or "improvements" to the purification process *upstream* of the immunogen selection point, *however minor they may appear to be*, require the approval of the purification process developer, quality assurance professionals, *and* the HCP assay developer. Identifying the difference between a "major" process change and a "minor" process change is sometimes a matter of serious contention. In many instances, regulatory agency approval should be obtained, as well.

The need for a decision to designate the purification process upstream of the prospective HCP immunogen selection point as "invariant" should be anticipated by Phase I, and implemented by Phase II, in order to allow enough time to have a process-specific HCP assay in place by Phase III [19]. This timing is particularly critical, since HCP assay development is expected to have a positive, iterative impact on purification process improvement and validation.

Because of the time and expense involved in completing these activities, it is important to avoid having to repeat them. Thus, it is essential that the null cell HCP immunogen preparation not be contaminated with the biopharmaceutical product which is usually purified by the process [51,52,61]. This situation may be avoided by any, or all, of the following: use of dedicated equipment which has not been exposed to the biopharmaceutical of interest; use of equipment which has been exhaustively cleaned and validated; or by contracting with a consultant firm for partial or complete manufacture of the null cell HCP immunogen in an outside facility. Two-dimensional silver-stained gel separations of prospective immunogen HCPs may be compared with duplicate immunoblots probed with a sensitive, and specific, immunoreagent for the biopharmaceutical product. This type of comparison can give reasonable assurance that the HCP immunogen is free of detectable contamination.

7. Anti-HCP immunoreagent development

In one conceptually simple case, S3 ribosomal protein, a unique, major HCP contaminant of recombinant human acidic fibroblast growth factor, was quantitated using specific monoclonal antibodies [54]. However, while this approach may be feasible for unusually simple immunogens, it would obviously be much more difficult to apply as the prospective HCP immunogen becomes more complex. There may be hundreds of potential HCP contaminants in a given immunogen preparation, and the individual HCP will differ widely in relative concentration and relative antigenicity. Thus, the practical goal becomes elicitation of a polyclonal immunoreagent which has demonstrable specificity, and sensitivity, for each of the antigenic proteins in the complex HCP immunogen. Using adjuvants, an efficient sequential immunization protocol, and a nominal boost-bleed cycle of 3 to 4 weeks, it may take 3–6 months to elicit, purify, and characterize an acceptable polyclonal anti-HCP immunoreagent against a very complex HCP immunogen. Any one of a variety of methods for purifying the IgG are acceptable. However, semi-preparative immunoaffinity chromatography (e.g. using Protein G) has been most efficient (this laboratory).

One must select an animal host which gives a strong immune response, and which yields appreciable amounts of immunospecific IgG. Specific pathogen free NZW rabbits are often a good choice. In order to minimize individual variations in immune competance, multiple animals (3–6) of a given species must be used, and

in some instances more than one species may be used.

Pragmatically, one must first assure a suitable immune response to the strong, or major, HCP antigens, and then induce a progressively enhanced response to the weaker, or minor, HCP antigens. The immune response to a limited number of HCP antigens may be relatively rapid, particularly when adjuvants are used to boost the immune response. However, in complex mixtures, differential enhancement of the immune response toward weaker, or lower concentration, antigens requires differential immunosuppression of major antigens. This may be accomplished by a couple of methods, either individually, or sequentially.

One method, passive immunization [55], involves purification of IgG from successive bleeds of immunized animals. The purified IgG is administered to the animals at the time of the next immunization along with the next dose of complete HCP immunogen. This IgG, which will have specificity against major antigens, tends to suppress further recognition of those same antigens in vivo. Immune recognition of, and antigenic response to, weaker antigens is promoted. This procedure may be performed at each boost-bleed cycle. Thus, the progressive immune response against weaker antigens is enhanced.

Another method, cascade immunization [56,57], also requires that immune IgG from successive bleeds be purified. However, this purified IgG is covalently affixed to a chromatographic support, and is used to immunospecifically adsorb major antigens from the HCP immunogen itself. The "adsorbed" HCP immunogen, which has been relatively depleted for major antigens in vitro, and thus relatively enriched for minor antigens, is administered in the next boost. This "antigen selection" facilitates immune recognition of, and response to, minor antigens. This procedure may be performed at each boost-bleed cycle. In this way, the immune response against progressively weaker antigens is enhanced.

To achieve true quantitation in complex, multi-analyte immunoassays, it is essential that the immunoreagent simultaneously specifically recognize, and be in stoichiometric excess to, all of the individual analytes of interest in an assay sample [50]. Demonstration of acceptable stoichiometry, and hence the ultimate acceptability of the anti-HCP immunoreagent, is strictly empirical. The progressive immune response achieved for a given immunization scheme may be monitored by using purified IgG from each sequential bleed to immunospecifically probe blots of HCP immunogen which has been adequately separated by electrophoresis. If the immunogen is simple, mono-dimensional electrophoresis [e.g. sodium dodecylsulphatepolyacrylamide gel electrophoresis (SDS-PAGE)] may be sufficient. If the immunogen is complex, two-dimensional electrophoresis (e.g. isoelectric focusing/SDS-PAGE) may be required to give optimal separation [41–43]. Ideally, all of the proteins detected by sensitive silver stain of an HCP immunogen electrophoretic gel should eventually also be detected in an immunoblot by the progressively enhanced anti-HCP immune IgG [57]. In practice, some minor proteins which elicit a strong immune response may not be visible in the silver stained gel, and some poorly antigenic proteins which are detectable by silver stain may never elicit a detectable immune response.

Anti-HCP immunoreagent specificity and stoichiometry are thus defined by, and limited to, those HCP immunogen proteins which have been demonstrated on blots to be specifically detectable by the immunoreagent. Sensitivity of the immunoreagent will ultimately be defined during assay development and validation, and should permit quantitation at ppm levels. Thus, the practical endpoint of anti-HCP immunoreagent development occurs when the investigator is satisfied that most of the HCP immunogen proteins are detectable by the immunoreagent, at an acceptable level of sensitivity.

Reagent stoichiometry may be further enhanced by immunospecific subpurification of specific anti-HCP IgG away from pre-existing IgG. For example, HCP immunogen may be covalently affixed to a chromatographic support and used as an immunoaffinity purification ma-

trix to obtain immuno-specifically subpurified anti-HCP IgG [58]. Microgram quantities of this pure reagent, used to detect nanograms to picograms of contaminant proteins in an individual assay sample would, by definition, be in stoichiometric excess [50]. In practice, an acceptable region, or range, of antibody excess in the standard curve of a given assay may be demonstrated empirically by spiked-addition (spike-recovery) studies, whether the immunoreagent is subpurified, or not [79].

Demonstration of stoichiometric immunoreagent excess for some assay samples may only be achieved by analyzing a dilution series [52,58,65]. It may be virtually impossible to achieve true antibody excess with the specific immunoreagent IgG *versus all proteins in a complex mixture* when attempting to assay upstream purification process aliquots which contain very high total HCP levels (D.V. Sinicropi; personal communication).

8. Examples of HCP immunoassays

Once suitable stoichiometry of the immunoreagent has been defined, HCP assay development is relatively straightforward. Several immunoassay methodologies have been employed for quantitative (and semi-quantitative) polyclonal HCP immunoassays. Examples include: an ELISA with nanogram level sensitivity for yeast HCPs in recombinant hepatitis B surface antigen vaccine [34]; an ELISA with ppm sensitivity for *E. coli* HCPs in recombinant human interferon gamma [58]; an ELISA with ppm sensitivity for *E. coli* HCPs in recombinant human growth hormone [52,59]; an ELISA for mammalian "cellular" HCPs (not media proteins) in recombinant human clotting Factor VIII:C [60]; an ELISA with ppm sensitivity for mouse fibroblast HCPs in recombinant human erythropoetin [61]; RIA/IRMA [62,63] or ELISA [64] with ppm sensitivity for *E. coli* HCPs in recombinant human insulin; an antigen-selected ELISA (ASIA) with ppm sensitivity for chinese hamster ovary (CHO) cell HCPs in a recombinant human tissue plasminogen activator [65]; and an "ultrasensitive" ELISA for quantitation of CHO HCPs in therapeutic recombinant human pancreatic DNAse I (D.V. Sinicropi; personal communication).

A semi-quantitative, monoclonal antibody immunoblot with 100 ppm discrimination for *E. coli* S3 ribosomal protein in recombinant human acidic fibroblast growth factor was previously mentioned [54]. A semi-quantitative, polyclonal, dot-blot immunoassay with 100 ppm discrimination has been developed for quantitation of *E. coli* HCPs in each of two recombinant malarial vaccine candidates which are purified by the same process [66].

The relatively recent introduction of immunoligand assay (ILA) technology [67] shows promise in shortening individual assay turnaround times per se, as well as for rapidly accelerating the pace of HCP immunoassay development in general. This is due to adaptation of a sensitive detection system [68] originally designed for generic quantitation of DNA at picogram levels [30], and to the introduction of commercially available IgG labeling reagents (Molecular Devices Corp., Menlo Park, CA, USA) which are compatible with the detector technology. Sensitive ILA methods have been developed for quantitation of CHO HCPs at ppm levels in recombinant human erythropoetin [69], for quantitation of *E. coli* HCPs at ppm levels in recombinant human basic fibroblast growth factor [70], for quantitation of *E. coli* HCP at ppm levels in recombinant human alpha interferon (M.A.C. Costello; personal communication), and for quantitation of residual *E. coli* HCP at ppm levels in recombinant bovine somatotropin (this laboratory [79]). ILA methods have been developed for rapid and sensitive quantitation of individual residual media proteins, affinity ligands, and immunoglobulins, as well (H. Merrick, personal communication; [75]).

Many other HCP immunoassays and immunoligand assays for quantitation of host cell or process contaminants have been developed and implemented in the biopharmaceutical industry in recent years. However, the proprietary nature of the purification processes upon which most of the recently developed process-specific HCP

assays are based, continues to preclude detailed descriptions in the open literature. As more biopharmaceuticals reach market this publishing situation should improve. In general, the thrust of current and future HCP immunoassay development is to shorten assay development timeframes, to shorten assay turnaround times, and to continue to improve analytical sensitivity below ppm levels.

9. Alternative assay methods

Currently there is no acceptable alternative to the use of highly specific multianalyte immunoassays to obtain quantitation of process-specific HCP in biopharmaceuticals, especially when ppm sensitivity for mixtures of proteins is required. Purely chromatographic separations have been inadequate except in the case of single impurities [54,71]. However, very recent developments in multidimensional chromatographic/electrophoretic separations, using post column reaction fluorescence detection, show great potential for use in sensitive, non-immunologically based HCP assay development [72–74]. This methodology is being aggressively pursued by leaders in the biopharmaceutical industry (D.V. Sinicropi, personal communication).

10. Conclusions

Quality control of residual HCPs in biopharmaceuticals is currently based upon a consensus triad of quality assurance analytical methods. This "regulatory package" of methods includes: silver-stained electrophoretic gels and immunoblots; process validation clearance studies based upon sound process design; and process-specific host cell protein assays. This triad of assurance, and the specific need to develop sensitive multi-analyte immunoassays for HCP, is specification driven. The desired approximate specification range for HCP for a given biopharmaceutical must be identified early in process development, and is related to the prospective use of the product. Often this specification will fall into a range of 1–10 ppm. It is prudent to include planning for development of a process-specific HCP immunoassay with part-per-million sensitivity into the earliest stages of product development. Timing is critical due to the prolonged time frames associated with the null cell mock purification model currently employed for multi-analyte HCP assay development.

In a practical sense, the triad of quality assurance methods are not mutually exclusive. While each method alone is insufficient to guarantee consistent removal of HCPs to desired levels, all three together give mutually supporting assurance. Anti-HCP immunoreagents generated for HCP assay development may also be used semi-quantitatively for early process optimization, or later quantitatively for process validation. HCP assay development can have a positive, iterative impact upon an evolving purification process, with respect to assuring consistency in achieving a desired specification range for the bulk drug.

Once process consistency for removal of HCPs to desired levels has been rigorously validated using the quality assurance triad, a specification assay for HCPs per se may not be required at all, or perhaps only on an audit basis. In this sense HCP immunoassay development may be thought of as a quantitative tool for validation of a consistent biopharmaceutical purification process, rather than as an end in itself. Combined qualitative and quantitative demonstration of process consistency for removal of contaminants is a natural extension of total quality principles [16,18]. These activities, in conjunction with properly designed pivotal toxicology and safety studies, should be suitable to formulate a persuasive contaminant specification rationale for any biopharmaceutical regulatory submission.

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