Defining Your Product Profile and Maintaining Control Over It, Part 2

Challenges of Monitoring Host Cell Protein Impurities

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In this second session of the CMC Strategy Forum (19–20 July 2004), representatives from the biopharmaceutical industry and the FDA discussed the challenges of monitoring host cell protein (HCP) impurities during the development and commercialization of recombinant protein drugs. Our discussion followed a session about process-related impurities in general. The sidebar provides more information about this meeting from Part 1 of this four-part article.

Recombinant protein pharmaceuticals are purified to a high degree from the host cells that produce them. Whether a recombinant protein is secreted from Chinese hamster ovary (CHO) cells, for example, or remains within a bacterial host (such as *Escherichia coli*), it is necessary for host cell derived proteins to be separated from the recombinant protein and for residual host cell proteins to be monitored in biopharmaceutical preparations. Because host cell derived proteins are highly complex mixtures of polypeptides, measuring them poses some challenging analytical dilemmas. Nonetheless, HCP assays are important tools for demonstrating product purity and consistency of manufacture.

PRODUCT FOCUS: ALL RECOMBINANT PROTEINS

PROCESS FOCUS: PROCESS DEVELOPMENT (PRODUCT CHARACTERIZATION)

WHO SHOULD READ: MANUFACTURING AND PROCESS DEVELOPMENT, PROJECT MANAGERS, AND ANALYTICAL PERSONNEL

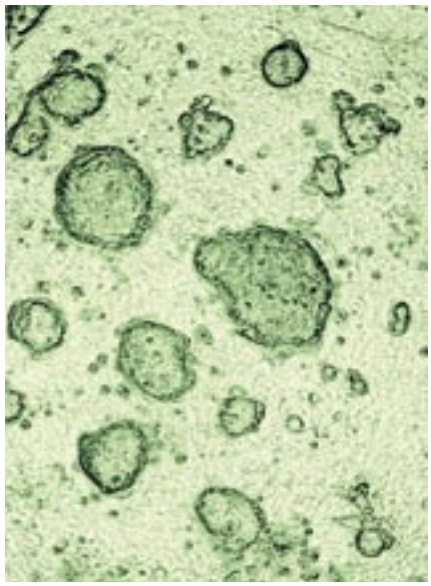
KEYWORDS: HOST-CELL PROTEINS, MICROBIAL AND ANIMAL CELLS, PROCESS-RELATED IMPURITIES, IMMUNOASSAYS

LEVEL: BASIC

MEASUREMENT BY IMMUNOASSAYS

Host cell protein populations are typically measured using multianalyte enzyme-linked immunosorbent assays (ELISAs) with polyclonal antibodies. The HCP values are expressed in units of ng/mL or parts per million (ng HCP per mg product). These values reflect the degree of specific immunoreactivity because they are a measure of the epitope population in a sample. Nevertheless, the value obtained in an ELISA is a reasonable measure of HCP mass, provided that the antibody reagent and immunoassay are characterized and can be shown to accomplish the intended purpose.

It is important to recognize both the limitations and strengths of using immunoassays to measure host cell proteins. The key limitation is that nonimmunoreactive or weakly immunoreactive proteins are not detected by these assays. To detect such proteins, an independent method is used, usually SDS-PAGE with a sensitive protein staining method (e.g., silver or SyproRuby). Some sponsors also use two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) to assess product purity.



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That method offers high resolution because it separates proteins by both isoelectric point and molecular mass. Application of 2D-PAGE may be useful in certain cases, but SDS-PAGE is generally sufficient. When bands are detected in polyacrylamide gels, Western blotting with antibodies to product and HCPs can be used in an attempt to distinguish product-related bands from HCP bands, respectively. The conditions used in Western blotting can be adjusted to allow binding by low affinity anti-HCP antibodies that do not bind in the ELISA format.

One strength of immunoassays is that they can detect host cell proteins at levels undetectable in gels. This is because an ELISA measures the collective sum of immunoreactive

Well-characterized biopharmaceutical products are defined during development by the identification and quantification, when at all possible, of both process-related and product-related impurities. However, expectations for the permissible levels of residual processing components or product variants throughout the product development life cycle are unestablished.

Several factors have contributed to the difficulty in standardizing those requirements, including safety of the residual components and process capability to reduce unwanted components. These, and other variables, have resulted in the "case-by-case" model for regulatory assessment of impurities to be specified and acceptable limits for control.

The absence of clear guidance contributes to uncertainty in the fitness of product development plans, potential misalignment of priorities with the true safety risk posed by each impurity, delay in regulatory review of filings as these factors are evaluated anew with each dossier, and inconsistent standards from product-to-product or sponsor-to-sponsor. Establishment of appropriate standards for acceptable levels of process-related and product-related impurities, along with strategies for removal and requirements for specification would facilitate efficient and cost-effective development, production, and availability of safe and beneficial new drug products.

The CMC Strategy Forum on Defining and Controlling Product Profile: The sixth Well-Characterized Biotechnology Pharmaceutical (WCBP) Chemistry, Manufacturing, and Controls (CMC) Strategy Forum was held on 19–20 July 2004 at the Lister Hill Auditorium on the NIH Campus in Bethesda, Maryland. Sponsorship of the event was provided by the California Separation Science Society (CaSSS; www.casss.org) as part of a series of discussions between industry and regulatory participants exploring current practices in analytical and bioprocess technologies for development and communication of consensus concepts.

The purpose of this two-day forum was to survey which methods are most useful in identifying and measuring process-related and product-related impurities — and identify strategies and specifications to ensure a consistent product profile. The first day concentrated on process-related impurities (Parts 1 and 2 of this article), whereas on day two the focus was shifted to product-related impurities (Parts 3 and 4).

proteins. In other words, a signal is obtained from a number of different proteins present at low levels. Therefore, even when a recombinant product is sufficiently pure that there is not enough of any particular host cell protein species to be detected in a polyacrylamide gel, an HCP ELISA can provide a measurement of residual host cell proteins. Overall, ELISAs are more sensitive than methods for monitoring individual HCPs except that they do not measure nonimmunogenic proteins.

Given the above considerations, a complete assessment of product purity with regard to host cell proteins should include information from more than one type of analysis. It should include the results of a meaningful ELISA as well as SDS-PAGE and possibly immunoblot analysis. Some sponsors have seen significant quantities of a particular host cell-derived protein present in recombinant protein preparations despite the lack of detection by ELISA. Detection of such impurities has been accomplished using PAGE or MALDI-TOF mass spectrometry.

Comparing Product-Specific and Multiproduct Immunoassays: Biopharmaceutical companies usually develop HCP immunoassays for their own use, maintaining proprietary assays and antibody reagents. Multiproduct ELISAs can be developed for all products derived from a particular cell type or expression sytem. For example, a sponsor may develop one assay for measuring CHO cell proteins and use it for all of that sponsor's CHO cell-derived products. Such multiproduct assays are acceptable, provided that both the antibody reagent and immunoassay are characterized and shown to be suitable for assessessing HCPs with each product. A broad spectrum of host cell proteins should be recognized by the antibodies. An antibody preparation that recognizes only a few proteins is insufficient for monitoring HCP populations and their removal during product purification. HCP populations differing widely from one product to the next would suggest that the multiproduct ELISA is not widely applicable, whereas similar HCP populations would suggest that it is.

The polyclonal antibodies used in multiproduct immunoassays are raised in moderate-sized mammals (typically rabbits or goats) against a complex mixture of host cell proteins, such as from a whole cell extract. Multiproduct assays differ from process-specific assays, which generally use antibodies against an in-process pool taken at the first downstream step in a purification process. Such pools are thought to represent the most likely impurities for a product rather than all the immunogenic host cell proteins that could be present. In some cases, such process-specific assays may be more sensitive or more specific than multiproduct assays.

Product-specific assays also have several shortcomings. For example, the applicability of an antibody preparation generated against partially fractionated material depends on the reproducibility of the manufacturing step(s) used to generate the HCP immunogen. Using an assay that is selective for host cell proteins that typically copurify with the product does not permit detection of those that may be present when a deviation

or change in the process has occurred. Similarly, if an HCP assay is developed early in product development, a new one will be required when the culture and recovery processes are finalized, and yet another may be required if postapproval process changes are implemented. Introduction of a new assay makes it difficult to compare HCP levels across different processes and therefore limits information concerning the manufacturing history of a product.

When using a multiproduct immunoassay, it is unnecessary to wait until the process is locked down before developing the residual HCP assay. Furthermore, using the same immunoassay for multiple products permits development of method expertise and consistent data interpretation. From a practical perspective, less training of personnel is required when one method is used rather than several methods. Reagent generation and maintenance are simplified and less expensive. Overall, both the process-specific and multiproduct approaches to HCP assay development are acceptable. The advantages and disadvantages of each should be considered before determining which one to select.

Acceptable Levels of Residual HCPs

Because host cell protein ELISAs are developed independently, and because the sensitivity and specificity of each assay depends on the host cell used and the way in which the assay was developed, ppm (parts per million) values obtained at one company are not necessarily equivalent to those obtained at a different company. The value provided by an ELISA indicates the collective sum of all immunoreactive HCPs present. It is not generally known whether that value is derived from the signals of 10 or 100 different proteins. So immunoblot analysis should be used as part of the development and assessment of all HCP ELISAs.

In contrast with their position on DNA, regulatory authorities have not set a global limit on host cell protein levels. Setting an acceptable level for residual host cell protein impurities is complicated by the different assays as well as the complexity of these impurity populations. Most biotechnology products reviewed by the FDA contain ELISA-based host cell protein levels of 1–100 ppm.

To date, only limited clinical adverse events have been attributed to HCP impurities. Nevertheless, there are general concerns about residual host cell proteins in biopharmaceuticals. Lingering safety concerns stem from the potential for adjuvant effects or allergic reactions to host cell proteins. In this regard, measuring HCP impurities using an ELISA is appropriate. An ELISA measures the HCP immunoreactivity in a sample and thereby may provide insight into potential immunoreactivity in humans. A Western blot immunoassay can be very useful (and sometimes necessary) to determining whether an immunogenic response in patients is against a residual HCP impurity or the product itself.

Several factors influence determination of an acceptable level of residual host cell proteins. For example, the species from which HCPs are derived is important because some cell types (e.g., yeasts) yield potentially more allergenic proteins than others. Additional factors include the manufacturing capability and history of a sponsor, the sponsor's ability to characterize the product, and the product safety profile. The drug dose must be considered because higher doses result in higher HCP loads to patients. The route (e.g., intravenous or subcutaneous) and schedule (e.g., acute or chronic) of administration for a drug product should also be considered when setting an acceptance level because they both significantly influence immunogenicity. For example, concerns about residual HCPs may be greater in a drug product administered in high, repeated doses subcutanously for a number of years than in one given in a single intravenous dose. Even though no major safety issues have arisen from residual host cell protein impurities, it is considered good practice to minimize HCP levels and thus limit the potential for unexpected adverse events such as molecular mimicry, anaphylaxoid reactions, and adjuvant effects. For these and other reasons, the FDA recommends that sponsors minimize the levels of residual host cell proteins in their products.

If a particular host cell protein copurifies with a therapeutic protein and can be detected by SDS-PAGE analysis, it should be identified and its level in the final product estimated and controlled. Identification of protein bands in gels is readily accomplished using peptide mass mapping or Edman degradation sequence analysis. The level of the HCP impurities should be estimated by conducting titrations with SDS-PAGE and/or immunoblot analysis, even though such analyses are only semiquantitative.

Similar levels of sensitivity are achieved by silver or SyproRuby detection (**1**). Whereas SyproRuby staining offers a much wider dynamic range than silver staining, that range is generally too narrow to provide relative levels of residual HCPs present. Although the nature of each particular HCP species is a factor to consider when setting an acceptable level, it is generally better to reengineer a purification process to remove that impurity to levels undetectable in gels. This is particularly true if the presence of host cell proteins hinders reliable characterization of the active pharmaceutical ingredient.

Comparing Multiproduct with Generic Immunoassays

When a sponsor develops an HCP immunoassay that can be used for all products derived from a given cell type (e.g., *E. coli* or CHO cells), that assay is considered a "multiproduct" assay. It is typically maintained as a proprietary assay with a proprietary antibody reagent. Such assays should be distinguished from generic assays. We use the term *generic* to refer to those assays developed by independent companies for use by a variety of biopharmaceutical sponsors. Theoretically, commercially available generic assays should be widely applicable to products made using a given cell type at different companies. To date, however, several sponsors have found insufficient sensitivity with such assays and a lack of immunoreactivity with certain host cell proteins.

Any immunoassay used to measure HCPs in biopharmaceutical preparations should be evaluated and proven capable of measuring numerous host cell proteins of various sizes that could potentially copurify with a product. Characterization of the antibody reagent should include SDS-PAGE immunoblots showing reactivity with numerous HCP bands. Assessment of a generic HCP assay should involve comparing a Western blot of the sponsor's own control sample with a standard from the commercial supplier (typically the immunogen used to produce the antibody reagents). Overall, generic (commercial) assays are considered acceptable by the FDA provided that they are properly qualified.

How Immunoassays Are Applied

HCP immunoassays are primarily used in the biopharmaceutical industry to demonstrate consistency of manufacturing processes and to characterize product purity. They can be used to monitor host cell protein levels at several stages in biopharmaceutical development and commercialization.

Early in drug development, immunoassays can aid in development and optimization of purification processes. These assays provide information about the ability of a new purification process step to remove HCP impurities. In fact, by using an appropriate HCP immunoassay early in drug development, a sponsor increases the utility of its manufacturing history.

As drug development progresses, HCP immunoassays are used during recovery process characterization to evaluate and identify process parameters that affect the ability of purification steps to remove HCP. Finally, HCP ELISAs are used to monitor HCP levels during recovery process validation, and they are often used as a release test for bulk drug substance. When used in process validation, they should be qualified to demonstrate their appropriateness. When used for drug substance testing, they should be validated. (Distinctions between and details of assay qualification and validation were not addressed in this session.) To ensure that an antibody reagent continues to recognize a broad spectrum of potential contaminants, its suitability for HCP detection should be reestablished following major manufacturing changes.

Process validation can be sufficient to replace a bulk drug substance specification for host cell proteins. ICH guideline Q6B on process-related impurities states, "For certain impurities, testing of either the drug substance or the drug product may not be necessary and may not need to be included in the specifications if efficient control or removal to acceptable levels is demonstrated by suitable studies" (**2**). Process validation and monitoring of host cell proteins is

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a widely used approach in which HCP immunoassays are used to validate the capabilities of a manufacturing process and demonstrate lot-to-lot consistency of a final product. However, until sufficient information on process capabilities and product characterization is available, a release test for HCPs in a drug substance is usually required. If HCP testing is not used for drug substance lot release, then HCP clearance should be reassessed following major manufacturing changes.

Use of Proteomic Studies

A series of proteomic studies was undertaken at Genentech to increase understanding of HCP populations that potentially enter purification processes and to assess the feasibility of multiproduct HCP ELISAs. These studies were initiated using *E. coli* host cells rather than CHO cells because the *E. coli* protein (ECP) population is theoretically simpler than that of a mammalian cell line. Furthermore, the *E. coli* genome has been completely sequenced, which makes identifying proteins by peptide mass mapping straightforward.

To assess potential differences between *E. coli* host strains and fermentation conditions used in various production processes, four products in development or on the market were selected for study (**3**). These host cell proteins were analyzed without interference from product-related proteins by performing control (blank) fermentations with host strains carrying plasmid vectors without the product genes. All host strains were derived from a common strain (W3110) and differed from one another by a few genetic markers (for details, refer to Reference 3). Cells were cultured under conditions that had been optimized for production of different recombinant proteins.Fermentations differed in duration, dissolved oxygen level, temperature, and other parameters. Upon completion of each fermentation process, cells were harvested and whole cell lysates analyzed by 2D-PAGE. The resulting proteomic profiles reflected the physiological state of the cells and the protein mixture that would potentially enter a purification process (**3**). Comparisons of these protein profiles revealed a high degree of similarity and revealed no dramatic differences in the protein and corresponding epitope populations from one *E. coli* host strain and culture condition to the next. This suggested that a multiproduct ECP ELISA was feasible.

To further investigate the applicability of multiproduct ECP immunoassays, *E. coli* cells grown with or without recombinant protein over-expression were compared. Control and production fermentations for human growth hormone (hGH) manufacturing were compared using silver-stained 2-D PAGE. The potential effect of fermentation scale on the host cell protein profile was also evaluated by comparing 10-L and 1000-L fermentations for human growth hormone production. As expected, the most striking distinction between the control and production fermentations was the large amount of hGH present in the production fermentations. An elevated level of a stress protein in the control

fermentation turned out to be due to an experimental artifact — namely, a truncated vector-encoded gene (**4**). Overall, neither the scale of fermentation nor overexpression of recombinant human growth hormone had a large impact on the host cell protein profile. The results of these proteomic studies suggest that multiproduct HCP ELISAs are not only feasible, but they can be readily applied across products generated by different strains and fermentation processes at different scales.

Finally, experiments involving 2D-PAGE Western blots and immunoaffinity fractionation were performed to evaluate immunoreactive ECPs. Results showed numerous immunoreactive ECPs that covered a broad range of molecular weights and isoelectric points. The results also suggested that there were high-affinity antibodies to certain *E. coli* proteins and low- or moderate-affinity antibodies to others. This was confirmed in a multiproduct ECP ELISA, and the details will be described elsewhere (**5**).

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