Why you should worry about process-related impurities in C&GT development

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In response to the many cell and gene therapy (C&GTs) products in development [1], the FDA and EMA recently published new guidelines for developers advising greater monitoring of process-related impurities. However, many C&GT developers experience problems removing contaminants and properly characterizing the product.

During the development and production of complex biologics such as cell & gene therapy products (C&GTs), process-related impurities co-purify along with the product.

The EMA Q6B guidelines [2] divide these impurities into three subtypes depending on their origin:

- Cell substrate-derived (host cell proteins, host cell DNA, etc.)
- Cell culture-derived (antibiotics, IPTG, DTT, growth factors, etc.)
- Downstream-derived (enzymes, buffer components, etc.)

The cell substrate-derived impurities include host cell proteins (HCPs), of which some are of concern if they are present in the final product. They include lipases, ubiquitin, peptidylprolyl isomerase, heat shock protein, cathepsin, and serine protease [3].

The cell culture-derived impurities include proteins added to the growth medium, e.g., fetal calf serum, human albumin, cytokines, and enzymes used for harvesting and lysing cells such as TrypLE.

Downstream derived impurities consist of agents used to express and purify biological protein products. These include the Benzonase nuclease, affinity column antibodies for product capture, enzymes for site-specific PEGylation, aminopeptidase, and Protein A. Other typical residuals are Tris, carriers, ligands, Tween/ Polysorbate, DCA, TCEP, heavy metals, solvents, Triton-X, antifoaming agents, PEI, TFA/Acetate, Imidazole, etc.

Why residual impurities are a concern

Investigating residual protein levels is crucial, especially in the final product, because the residuals can influence the stability and efficacy of the active ingredient and may also pose a risk to the patient's health.

Such 'problematic' proteins may:

1. Affect drug efficacy by reducing protein stability and potency, and/or
2. Induce immunotoxic effects and immunogenic reactions in patients

-even in sub-ppm amounts.

The foreign, residual proteins increase the risk of triggering an immune response in the patient receiving the treatment.

Most host cell proteins (HCPs) have the potential to generate an immune response in humans due to the genomic differences between the human patient and commonly applied protein production hosts, such as E. coli, yeast, mouse myeloma cells (NS0), and Chinese Hamster Ovary cells (CHO). For example, Wang et al showed that a reduction in HCPs correlated with a decline in the release of specific inflammatory cytokines [4].

There is also a risk that some unwanted HCPs, similar to human homologs, may have a biological function in humans resulting in potential side effects [4].

To summarize, it is essential to monitor residual proteins since they influence:

- The product quality by proteolysis, particle formation, or enzymatic modification;
- The process consistency and, therefore, the ability to produce a pure product;
- The patient safety by inducing immunogenicity or decreasing activity of the biologic's active ingredient.

With the development of sensitive analytical methods for residuals, the FDA and EMA are increasingly interested in data describing the efficiency of the purification process for C&GTs.

Hence, an analytical method is needed to detect the exact HCPs that appear in your product – throughout process development and in the final drug substance.

It is challenging to monitor problematic HCPs using ELISA

The FDA increasingly ask C&GT developers to document residual protein clearance before the biologic goes into late-stage clinical trials [5].

The problem is that traditional methods like ELISA and HPLC are limited by not being able to distinguish between impurities (low resolution) and insufficient sensitivity (limit of detection), respectively. Since the residuals are present at low-ppm levels, you need a reproducible approach with a high sensitivity to get a detailed overview of the residual protein clearance [6-7].

Furthermore, since the products are complex and contain proteins from multiple sources and species, a standard commercial ELISA cannot measure all these impurities. An alternative is to develop a process-specific ELISA to cover the

HCPs in these products, but this is costly, time-consuming, and challenging.

Orthogonal method for analysis of residual protein impurities in gene therapies based on adenovirus

A typical challenge is the detection of protein residuals in products based on adenovirus expressed in a human cell line, e.g., HEK293 or A549 cells, grown on a cell-substrate containing bovine serum albumin.

Adenoviruses consist of a protein capsid with different proteins that enclose the DNA and core proteins (see the figure above). When the virus is reproduced and purified from the human host cells, the virus drug substance contains small amounts of residual human proteins. Therefore, the high number of different proteins in the drug requires characterization by a highly sensitive method before clinical administration [8-10].

The best analysis method for this mixture is SWATH LC-MS (liquid-chromatography mass spectrometry), based on data-independent acquisition (DIA), as it can both quantify and identify protein impurities. The LC-MS analysis is highly reproducible and robust and thus suitable for residual protein and HCP analysis.

The analysis provides A) the total amount of residual host cell proteins in ng/ml, B) a list of identified residual proteins and their amounts, and C) a list of identified viral proteins and their amounts.

Mass spectrometry is not relying on animal immune responses and can identify and quantify individual proteins. It is ideal for documenting proteins from different expression organisms that induce process-related impurities in complex C&GT biopharmaceuticals [11-12].

Benefits of using SWATH LC-MS for analyzing residual proteins in C&GTs

- Highly reproducible identification and quantification due to the use of DIA mode.
- Interference, from a high amount of drug substance on the signal of low abundant residuals, is kept down by using small mass windows for MS/MS fragmentation.
- Compare batches and use it as a tool for quality control after process scale-up.
- High throughput sample handling makes it possible to assess residuals in various manufacturing steps.
- High sensitivity makes it possible to quantify low ppm levels.

With these advantages, SWATH LC-MS is ideal for analyzing process-related residuals used in biologics development.

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