400
300
200
100

Vetoing the Virus

Dr Andy Bailey at ViruSure GmbH investigates current issues in the validation of biopharmaceuticals for virus clearance

Dr Andy Bailey has been actively involved in the pathogen safety of biopharmaceuticals for over 11 years. Originally a Biochemist, Andy served for nine years at the MRC Virology Unit in Glasgow, Scotland. In 1995, he moved as Director of Virus Validation services to Q-One Biotech Ltd, and in 2001 to the Pathogen Safety group of Baxter Healthcare in Vienna, Austria. Over the last 10 years, Andy has presented at numerous regulatory agencies, either in support of products or as an invited speaker at expert workshops, including the UK MHRA, German PEI, French AFFSAPS, US FDA, EMEA and JMHLW (Japan).

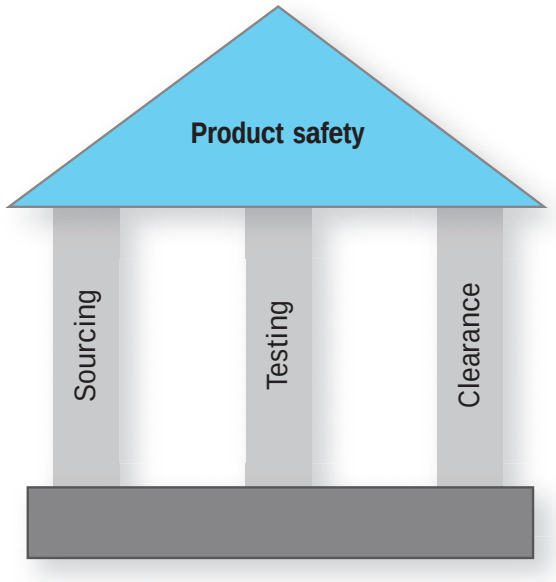
Recent years have seen a shift in the paradigm for virus safety, away from an assumption that each pillar of the safety triangle (see Figure 1) contributes equally to the overall virus safety profile of a product, towards a view that virus inactivation and/or removal may play a more important role in assuring the safety of the product (1). The degree to which this is apparent is product dependent, and this shift in paradigm has been most noticeable in the human plasma products industry. The actual contribution to risk reduction by donor selection, donor screening and virus inactivation/removal can be mathematically modelled and shows that donor screening and donor testing each contribute in the order of a 1-2 log10 reduction in measurable risk for viruses such as HIV or HCV (1,2). By contrast, the incorporation of two steps into the manufacturing process, each providing in the order of 5.0 log10 inactivation or removal, can provide a risk reduction in the order of 10 log10. Such data has resulted in ever increasing scrutiny of the manufacturing process, in particular ensuring that the design of the virus inactivation study and presentation of the data is such that the reduction factors claimed for a manufacturing process can be relied upon. This review looks at a number of aspects of virus clearance study design that have proven critical in ensuring that the design and interpretation complies with current regulatory requirements.

HOW MANY VIRUS INACTIVATION/REMOVAL STEPS DO I NEED?

This question can only be answered by first understanding the nature of the product. It is almost certain that any manufacturing process that includes two to three dedicated and effective virus inactivation/removal steps is likely to meet even the most stringent of regulatory reviews. The extent to which a process will be viewed as having sufficient viral clearance will depend on the various strategies adopted during the design phase of the product. A product which completely excludes components of animal origin is likely to require less virus removal in order to pass regulatory scrutiny than a product that still uses components of animal origin. The nature of the animal-derived components and the potential virus contaminants may also impact on any assessment of how much virus clearance will be considered sufficient. Where animal-derived components are used, it is advisable to seek virological expert advice regarding potential concerns for virus contamination.

The question of how much clearance is sufficient is also dependent on the nature of the virus clearance steps incorporated (effective against enveloped viruses, non-enveloped viruses or both). Steps such as solvent/detergent treatment for the inactivation of enveloped viruses have a long history and are well accepted (3). Other steps may not be viewed as providing a similar level of assurance. Chromatography or precipitation steps, for example, contribute to virus removal, but

Figure 1: The safety tripod for virus safety



processes that rely solely on partitioning steps have historically resulted in a higher probability of virus transmission (4). Dedicated virus clearance steps with an established history of effective virus removal should therefore be planned into the process to allay any virus safety concerns. The validation of new technologies for virus clearance will require more data in order to convince authorities of the robustness of the step. A list of established and emerging technologies for virus inactivation or removal is provided in Table 1.

Table 1: Established, contributing and emerging virus removal and inactivation technologies

Established effective virus removal/inactivation technologies	Solvent/detergent Nanofiltration* Low pH (≤4.0) High pH (≥12.0) * Heat (pasteurisation) * Dry or vapour heat*
Contributing partitioning steps	Chromatography Precipitation Depth filtration Charged separation filters
Emerging inactivation removal technologies	UV inactivation* γ-irradiation* Chemical inactivation*

* Steps potentially effective for the inactivation of enveloped and non-enveloped viruses

Non-enveloped viruses tend to be more difficult to inactivate or remove than enveloped viruses (5-7). Non-enveloped viruses also tend to be smaller, making them more of a challenge for size-based removal (such as nanofiltration). Manufacturing processes that fail to provide for effective removal non-enveloped viruses will inevitably receive more questions about measures for controlling risk from such viruses. It is therefore advisable to plan for at least two dedicated virus inactivation/removal steps, at least one of which should be effective against both enveloped and non-enveloped viruses. Steps effective against both class of virus are highlighted in Table 1.

GENERAL PROCESS DESIGN CONSIDERATIONS

The positioning of a given virus clearance step within a manufacturing process may impact on the effectiveness of the step to clear virus. Additionally however, the implementation of dedicated virus inactivation/removal steps impacts on the overall facility design. Facilities must be designed to ensure adequate segregation between product that has been treated for virus removal and product which has not yet been treated. Inadequate segregation is a frequent observation during facility inspections, and thus care must be taken to ensure appropriate design of the process and facilities. Separate rooms are not necessarily required to ensure segregation, and in some instances it may be possible to provide sufficient segregation using closed tank systems. A detailed review of facility design and segregation is, unfortunately, outside the scope of this review.

GENERAL CONSIDERATIONS IN VIRUS CLEARANCE STUDY DESIGN

The design of virus clearance studies is the subject of numerous regulatory guidelines (8-10). It is therefore perhaps no surprise that virus removal during the review process receives significant scrutiny. The following sections review some of the areas that frequently lead to questions and/or additional work prior to acceptance of a submission dossier.

Cytotoxicity and Interference Studies

Virus assays are sensitive biological systems, and are easily influenced by the matrix in which the virus is titrated. It is important therefore, to control for any potential impact of the matrix on virus titre. For samples spiked with a high titre of virus, the sample itself will be diluted significantly before the end-point virus titre is reached, and at such dilutions any potential impact of the matrix on virus titre is likely to be negligible. Where present, interference will be observed for samples containing low concentrations of virus, and thus interference testing should be designed to investigate possible effects at or around the end-point titre of the virus. A failure to appropriately investigate interference at or around the end point may result in over-inflated virus reduction factors.

Influence of the Virus Spike on the Down-scale

Virus spikes are routinely prepared in medium containing foetal bovine serum. It is advisable therefore to perform control mock-spiked experiments to assess any potential impact of the spike material on the performance of the down-scale. One recommendation is to combine such experiments with validation experiments demonstrating the validity of the downscale, which will in any event be required in support of the virus clearance study.

Mass Balance

Mass balance in virus clearance studies is a requirement detailed in regulatory guidance for the performance of virus clearance studies (8), but it's usefulness can be readily disputed. In many virus clearance studies, the input virus may not be quantitatively recovered in other fractions, even for processes where little or no virus inactivation occurs. The reasons for this are not well understood, but may include, for example, virus remaining bound to a column, virus aggregation (resulting in a lower titre upon titration) or assay variability for the titration system (for example, the 95 per cent confidence intervals for most virus assays are in the order of 0.3-0.5 log10). The value, therefore, of mass balance data has been questioned, and such data can only be used to make generalised statements about where the virus is partitioning, and should not, as is often the case, be over-interpreted to claim that 'all' virus was recovered when in fact the accuracy of the virus assays preclude any such claims.

Robustness

The robustness of any virus removal/inactivation step is today a critical component in the design of the virus validation study.

The issue of robustness has been slightly confused by different definitions given in the CPMP Note for Guidance on Virus Validation Studies (8), and that given in the ICH Q5A guidance document on the Viral Safety Evaluation of Biopharmaceutical Products (10). The CPMP document defines robustness as "...parameters which influence the effectiveness of a process step to remove/inactivate viruses", whereas the ICH document defines robustness in reference to the ability of the manufacturing process in general to remove specific or non-specific model viruses. For the purposes of this paper, robustness is used to refer to parameters which influence the effectiveness of a process step to remove/inactivate viruses, although the value of the ICH definition in the context of designing a robust process for virus removal should not be disregarded.

The robustness of a given virus clearance step provides important insights into the extent to which a process step can be relied upon to consistently provide a given level of virus clearance. Steps for which the level of virus clearance is unaffected by process parameters will provide for a higher level of assurance that the claimed level of virus removal/inactivation will be consistently reached in each manufacturing run. Inadequate investigation of robustness remains a frequent observation by authorities, and needs to be carefully considered prior to the study design if possible delays to licensure of the product are to be avoided. Specific aspects critical to the robustness of some of the more established technologies are discussed in more detail in the following sections. For inactivation steps, the kinetics of inactivation remains the most important information regarding the robustness of the step (3,5). Inactivation steps that provide for rapid and complete inactivation, or complete inactivation within 50 per cent of the total exposure time, will provide a higher level of assurance than inactivation steps where virus is still detectable out to near the end of the process incubation.

SPECIFIC CONSIDERATIONS FOR ESTABLISHED VIRUS CLEARANCE STEPS

Solvent Detergent

For studies investigating the solvent detergent step, a number of specific considerations are important both in respect to how and where it is implemented in the manufacturing process, as well as in respect to the design of virus inactivation studies (3). Regulatory guidance for solvent detergent steps requires that the product is free of gross aggregates which might protect the virus from inactivation (11). Operatively this is often performed following the addition of solvent/detergent reagents, which ensures the removal of both aggregates present prior to addition of the S/D mix, as well as any aggregates formed as a result of S/D addition (that is, protein aggregation). Filtration of the material into a separate clean tank (or through a dedicated line into a segregated room) also serves to ensure that all product is exposed to the S/D reagents, and that the potential for contamination with untreated product is minimised.

In the validation of S/D steps, it is important to ensure that S/D reagents are effectively neutralised immediately upon sample collection and prior to titration. This is most easily accomplished by dilution of the sample upon collection, although alternative methodologies have been explored (such as C18 resins (12)). Whichever method is employed, appropriate controls must be incorporated into the virus clearance study to demonstrate effective neutralisation of the S/D reagents, and that the neutralisation procedure itself does not result in a loss of virus titre.

At standard S/D concentrations, virus is rapidly inactivated such that it is often impossible to detect virus even at the first time-point tested. A frequent request of licensing authorities has been to investigate inactivation under non-standard conditions (five or 10 per cent of manufacturing concentrations). Although data generated at 10 per cent of manufacturing conditions may result in virus detectable at one to five minutes following S/D addition, it is clear that such data is not applicable to standard manufacturing conditions. It is recommended, however, to include such studies in order to allay any potential questions later in the licensing procedure.

Nanofiltration

Nanofiltration has become one of the most common virus clearance unit operations in bioprocessing (13-19). In particular, small pore filters (nominal pore size ~20nm or less) have become increasingly popular, and fulfil the requirement of an effective step for removal of even the smallest of viruses. For licensed products, they also offer the possibility of a process upgrade with the lowest probability for additional clinical trials prior to approval of the process variation. The increasing use of nanofiltration in biopharmaceutical manufacture has consequently resulted in an increased scrutiny of virus removal data.

Theoretical assumptions for nanofiltration require that removal is based primarily on size, that membrane polarisation is minimal and that the product matrix does not significantly impact on virus removal capacity. In practice, membrane polarisation is matrix dependent and does occur and can impact on virus removal. Robustness has therefore become a critical component in the design of nanofiltration viral clearance studies, and is essential in convincing authorities that the filter is able to reproducibly and effectively remove viruses. Robustness studies for nanofiltration are of most value when performed using a virus close to the pore size of the filter. For this reason, Parvoviruses are most frequently selected for studying the robustness of removal by filters of ~20nm or less (17,19). For larger pore size filters, robustness studies should be performed with a virus that is close to, or slightly larger than, the pore size of the filter. Studies performed with viruses significantly larger than the pore size of the filter are also required, but robustness studies should focus on the smaller viruses included in the study.

One of the most important aspects of nanofiltration study design is the aggregation status of the virus. Virus aggregation

may be virus-specific (e.g. present even prior to spiking) or induced as a result of spiking (matrix induced virus aggregation – through virus specific antibodies, for example). Irrespective of the cause, steps should be included to ensure that any virus aggregates are removed from the system prior to applying the spiked product matrix onto the nanofilter. A failure to remove any virus aggregates larger than the nominal pore size of the filter will result in overestimation of the virus removal capacity of the filter. Pre-filtration of the virus spiked product solution through a filter with a pore size slightly larger than the size of the virus (such as for a parvovirus pre-filtration through a filter with a pore size between 30 and 50nm) adequately controls for virus aggregation. Such a pre-filtration may also serve to remove any potential protein aggregates introduced via the virus spike, and which can often lead to premature filter blocking and difficulties in the interpretation of nanofiltration data.

Chromatography

Chromatography is frequently validated for virus removal, and in many instances contributes significantly to virus removal, supplementing virus clearance afforded through dedicated virus inactivation/removal steps. The down-scaling of chromatography steps, however, needs to be carefully controlled and validated prior to proceeding with any virus removal studies. Any downscaling issues or changes to the process identified at a later stage will almost inevitably result in having to repeat the virus removal studies. Robustness of chromatography steps is being increasingly requested by reviewing authorities and parameters such as pH, protein load and conductivity should be carefully evaluated to determine if additional studies to provide robustness data are required. Data generated during process validation can often be useful in identifying parameters that might potentially impact on virus removal.

CONCLUSION

The design of virus clearance studies has become a subject of increasing scrutiny during the regulatory review of biopharmaceutical products. This is reflected in the importance that virus clearance operations now hold in the overall process design for biopharmaceutical products. Ensuring the correct design of virus clearance studies has therefore never been more important, and can help to avoid potentially costly delays in the regulatory approval of biopharmaceutical products. ◆

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