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Inactivation of adenovirus types 5 and 6 by Virkon® S

Lisa McCormick, Gargi Maheshwari*

Fermentation and Cell Culture, Bioprocess R&D, Merck Research Laboratories, Merck and Co., Inc., P.O. Box 4, WP17-201, West Point, PA 19486, USA

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Abstract

Throughout the pharmaceutical industry, adenovirus-based products are being developed for human use as vaccine vectors and gene therapy delivery vehicles. The implementation of effective decontamination procedures is critical to the successful manufacture of these products to minimize the risk of personnel exposure and prevent product cross-contamination in the manufacturing facility. In this investigation, we have conducted small-scale decontamination studies to determine the efficacy of Virkon® S on the inactivation of adenovirus types 5 and 6 in suspension. Virkon® S is a commercially available oxidative disinfectant used against a variety of bacteria, spores, fungi, and viruses. A cytotoxicity-based endpoint dilution assay was used to quantify adenovirus potency before and after Virkon® S treatment. We show that the level of organic content in the inactivation sample matrix has a significant impact on Virkon® S activity. The potency of adenovirus types 5 and 6 was reduced by greater than six logs upon a five minute exposure to the appropriate concentration of Virkon® S. Based on these results, we propose that Virkon® S liquid decontamination procedures for adenovirus types 5 and 6 use 0.9% Virkon® S for contact times greater than five minutes.
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Keywords: Adenovirus; Decontamination; Inactivation; Virkon® S; Potency assay

1. Introduction

Throughout the pharmaceutical industry, adenovirus-based products are being developed for human use as vaccine vectors and as gene therapy delivery vehicles (Limbach and Paoletti, 1996; Zhang, 1999; Nadeau and Kamen, 2003). Owing to the high commercial potential of such products, large manufacturing facilities are being designed for their production. The assessment and implementation of effective decontamination procedures is critical to the successful large-scale manufacture of these adenovirus-based products. In recent years, regulatory agencies have emphasized the significance of quality assurance measures in the manufacture of biopharmaceuticals, including the execution of viral inactivation studies during process validation (White et al., 1991; Luff, 1992; Larzul, 1999).

First and foremost, the implementation of effective decontamination procedures minimizes the risk of personnel exposure to infectious agents (Sofer, 2003). In addition, the rising cost of operating a biological manufacturing facility and an increasing prevalence of multivalent products have resulted in the design of multi-product facilities, which in turn require stringent decontamination procedures to prevent product cross-contamination (Sofer, 1995; Doblhoff-Dier and Bliem, 1999). To design adequate viral inactivation procedures for the large-scale manufacture of adenovirus-based products, it is essential that small-scale studies be performed to demonstrate their efficacy.

In this study, we have examined the inactivation of adenovirus types 5 and 6 in suspension by treatment with Virkon® S (Antec™ International, Sudbury, Suffolk, UK; a division of DuPont™, Wilmington, DE), a commercially available oxidative disinfectant used against a variety of bacteria, spores, fungi, and viruses. A large collection of independent Virkon® S efficacy studies can be accessed through the manufacturer (www.antecint.co.uk). While very limited information on Virkon® S efficacy has been documented in the scientific literature, a series of investigations have been performed to test the efficacy of Virkon®, another disinfectant with the same active ingredient and a similar composition as Virkon® S (Gasparini et al., 1995; Angelillo et al., 1998; Hernandez et al., 2000). The composition of Virkon® S is described as a balanced, stabilized blend of peroxygen compounds, surfactant, organic acids, and an inorganic buffer system. In solution, Virkon® S is activated to form hypochlorous acid (HOCl), which has been shown to be a

* Corresponding author. Tel.: +1 215 652 6756; fax: +1 215 993 4884.
E-mail address: gargi_maheshwari@merck.com (G. Maheshwari).

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strong oxidizing agent (Spickett et al., 2000). The use of Virkon® S can be implemented easily and safely in a manufacturing facility, as it exhibits low ecotoxicity, is highly biodegradable, and is not corrosive to stainless steel. In addition, a 1% solution of Virkon® S is classified as a non-irritant to the skin and eyes (www.antecint.co.uk).

In this paper, we have implemented a cytotoxicity-based endpoint dilution assay to quantify adenovirus infectivity levels before and after Virkon® S treatment. The equivalency of the endpoint dilution assay to plaque titration was established independently during assay development. This endpoint dilution assay permitted quantitation of viral potency at levels as low as 30 infectious units per milliliter (IU/mL), which is the assay limit of detection, without interference from the sample matrix. In this report, we demonstrate that the degree of adenovirus inactivation is independent of the adenovirus construct or serotype. We show that the level of organic content in the sample matrix has a significant impact on Virkon® S activity. The potency of adenovirus types 5 and 6 was reduced by greater than six logs upon a five-minute exposure to the appropriate concentration of Virkon® S.

2. Materials and methods

2.1. Cell culture

A human embryonic kidney cell line expressing the E1 region of adenovirus type 5, 293 cells (Microbix, Toronto, Canada), were cultured in MEM-α medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT), penicillin (100 units/mL), and streptomycin (100 μg/mL) (Mediatech, Herndon, VA). Cells were planted at a density of 5.8×10^3 cells/cm^2 in vented T-150 flasks (Falcon, Bedford, MA) and incubated at 37 °C with 5% CO_2 in a humidified incubator. Cells were passaged every seven days using 0.25% (v/v) trypsin-citrate (Merck Manufacturing Division, West Point, PA) and were maintained for a maximum of five passages.

2.2. Endpoint dilution assay

Briefly, 293 cells were cultured in 96-well tissue culture plates (Falcon, Bedford, MA) at a density of 3.16×10^4 cells/cm^2 using MEM-α medium supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were maintained in 96-well plates in a humidified incubator at 37 °C with 5% CO_2 for approximately 48 h prior to infection. Infections were performed by removing culture medium from 293 cell monolayers and dispensing virus at the appropriate dilution into each well. After virus addition, plates were stored in a humidified incubator at 37 °C and 5% CO_2 for approximately two hours to allow virus to adsorb onto the cells. Medium containing 10% FBS was then added into each well and the plates were incubated in a humidified incubator at 37 °C with 5% CO_2 for 12 days to allow development of cytopathic effect. At 12 days post-infection, cell viability in each well was measured by staining with Cell Titer 96® Aqueous One Solution (Promega, Madison, WI). The absorbance of each plate at 490 nm was then read using a SpectraMax® Plus plate reader (Molecular Devices, Sunnyvale, CA). A minimum of 60 wells were inoculated with medium containing 10% FBS as negative controls. A sample well with a spectrophotometric reading less than 50% of the negative control median value was considered positive for adenovirus infection. This 50% cutoff value was established by independent method validation studies which demonstrated a correlation between the endpoint dilution assay and a quantitative PCR-based infectivity assay (Maheshwari et al., 2004). Sample titer was then calculated assuming a single infectious particle per positive well. A sample titer was calculated only when fewer than 50% of the wells at a given dilution scored positive. The equation used to calculate sample titers generated from the endpoint dilution assay is shown here.

$$\text{Endpoint dilution assay titer} = \frac{\#\text{wells positive for adenovirus infection (IU)}}{(\text{infection volume/well}) \times \text{wells infected}} \times \text{sample dilution factor}$$

In order to evaluate the variability of the endpoint dilution assay, the assay standard deviation was calculated using the log transformed values of the reported titer of a single untreated adenovirus sample which had been run in 21 independent assays. Using these data, the standard deviation was determined to be $\log_{10}$ 0.27 infectious titer. The 95% confidence interval for the titration of this untreated sample was found to be ± 0.57 $\log_{10}$ infectious titer (Michelson and Schofield, 1996). This potency assay has a limit of detection of 30 IU/mL, which is the titer that would be measured if only a single positive well was detected.

2.3. Selection of virus samples for investigation

The inactivation profiles of three adenovirus type 5 constructs with unique DNA sequences were evaluated in this study. These replication-incompetent constructs were generated by replacing the E1 gene of an adenovirus type 5 vector with various HIV-1 transgenes (Shiver and Emini, 2004). The 293 cells used to titer virus samples in the endpoint dilution assay have been transformed by the E1 region of human adenovirus type 5, allowing virus replication and development of cytopathic effect. Following the detailed analysis of Virkon® S efficacy against multiple adenovirus type 5 constructs, a single adenovirus type 6 construct was tested to demonstrate the impact of varying the adenovirus serotype. To evaluate the effect of a range of physicochemical parameters such as the presence of process buffers and varying protein and nucleic acid concentra-

tions on the efficacy of Virkon® S inactivation, virus samples from two different steps of an adenovirus production process were selected for analysis. The first sample type tested was purified adenovirus, which contained the lowest available protein and nucleic acid concentrations, and was formulated in stabilizing buffer to minimize viral infectivity loss during long-term storage. The second sample type tested was concentrated cell lysate from adenovirus-infected cells (crude virus), which was used to determine if high concentrations of protein and nucleic acids in the sample matrix had an effect on adenovirus inactivation. When compared to the purified samples, this crude virus was found to contain greater than 50-fold higher protein concentrations (as measured by a colorimetric protein quantitation assay) and greater than 100-fold higher nucleic acid concentrations (as measured by a fluorescent nucleic acid staining method).

2.4. Virkon® S inactivation procedure

A 2% (w/v) Virkon® S (Antec™ International, Sudbury, Suffolk, UK, a division of DuPont™, Wilmington, DE) solution was prepared from powder at room temperature on the day of sample preparation. 1 and 0.1% Virkon® S solutions were prepared by serially diluting the 2% stock. To initiate inactivation, virus samples were diluted 10-fold into the appropriate disinfectant concentration. After incubation for the desired time, the oxidative activity of Virkon® S was stopped by sodium thiosulfate (LabChem Inc., Pittsburgh, PA) addition, followed by pH neutralization by sodium bicarbonate (Sigma-Aldrich, St. Louis, MO) addition (target pH range was 6.8–7.4). Each neutralized sample was subsequently vortexed, spun down using a low-speed benchtop microcentrifuge (to collect the treated sample at the bottom of the tube), and diluted in stabilizing buffer to minimize viral infectivity loss during freezing and storage at −70 °C. Samples were thawed at room temperature prior to analysis by the endpoint dilution assay.

Spike recovery analysis was performed to examine the efficacy of the neutralization conditions selected for Virkon® S inactivation. Complete neutralization of Virkon® S was confirmed by spiking untreated virus into neutralized Virkon® S (for a final concentration near the assay limit of detection, 30 IU/mL) at all disinfectant concentrations tested. In addition, experiments were performed to investigate the reproducibility of the endpoint dilution assay in the presence of the inactivated sample matrix, which contained adenovirus, Virkon® S, and the chemicals used to neutralize Virkon® S activity. Untreated virus was spiked into dilutions of purified and crude adenovirus samples inactivated by Virkon® S treatment (for a final concentration near the assay limit of detection, 30 IU/mL). The virus titer recovered from these experiments was compared to its corresponding untreated control titer to detect the presence of incomplete disinfectant neutralization or assay interference from the sample matrix.

2.5. Calculation of log reduction values

Log reduction values (LRV) observed at a given Virkon® S inactivation condition were calculated by taking the logarithm (base 10) of the ratio of the infectivity of an untreated virus sample spiked into the neutralized sample matrix to the infectivity of the same sample following inactivation treatment. If the sample potency fell below the assay limit of detection, the LRV was calculated using the assay limit of detection and was reported as greater than that value.

3. Results

3.1. Neutralization of Virkon® S activity

Before conducting inactivation studies to test the efficacy of Virkon® S against adenovirus, a disinfectant neutralization scheme was developed. Sodium thiosulfate was identified as a potential neutralization agent, as it was previously used as an agent to neutralize the activity of Virkon® (another disinfectant manufactured by Antec International, with the same active ingredient as Virkon® S) (Gasparini et al., 1995; Angelillo et al., 1998). To test the efficacy of Virkon® S neutralization by sodium thiosulfate, adenovirus samples were spiked into different concentrations of Virkon® S that had been subjected to various neutralization treatments. It was quickly observed that, while sodium thiosulfate (at a final concentration of approximately 19 mM) might be neutralizing Virkon® S activity, it was not effective at neutralizing the acidic pH of Virkon® S (the pH of a 1% Virkon® S solution is 2.6). Therefore, sodium bicarbonate was also added (at final concentrations ranging from 9 to 42 mM, depending on the sample matrix) to accomplish pH neutralization. To test the efficacy of this neutralization strategy, untreated virus was spiked into neutralized Virkon® S (for a final concentration near the assay limit of detection, 30 IU/mL) at all disinfectant concentrations tested. Neutralization conditions were deemed successful when no statistically significant differences were observed between the virus titer recovered from the neutralized Virkon® S solution and its corresponding untreated control titer within the variability of the endpoint dilution assay (data not shown).

Experiments were also performed to investigate the consistency of the endpoint dilution assay in the presence of the inactivation sample matrix. The absence of assay interference was confirmed when no statistically significant differences were observed between the titer of adenovirus type 5 recovered from the inactivated sample matrix and its corresponding untreated control titer within the variability of the endpoint dilution assay (Table 1). Based on the sample dilutions tested in this analysis, successful spike recovery also demonstrates that the endpoint dilution assay can quantify viral potency at levels as low as approximately 30 IU/mL in a background of greater than 1×10^6 inactivated virus particles per milliliter.

Table 1
Spike recovery of purified and crude adenovirus type 5 in the inactivation sample matrix

Sample type	Sample description	Treatment condition	Spike Recovery in inactivation sample matrix (%)
Adenovirus type 5	Purified, construct 1	0.09% Virkon® S	187.1
	Unpurified, construct 1	0.9% Virkon® S	105.8

To confirm the absence of assay interference from the inactivation sample matrix, untreated, purified adenovirus type 5 was spiked into a sample of purified virus which had been subjected to 0.09% Virkon® S treatment for five minutes. Likewise, untreated, crude adenovirus type 5 was spiked into a sample of crude virus which had been subjected to 0.9% Virkon® S treatment for the same time period. Sample potency was then assayed using the cytotoxicity-based endpoint dilution assay (see Section 2). Spike recovery was calculated by determining the ratio of adenovirus type 5 potency recovered from the inactivated sample matrix to its corresponding untreated control titer.

3.2. Effect of sample matrix on Virkon® S inactivation

Adenovirus may be present in different matrices throughout the course of a manufacturing process. To evaluate the impact of sample matrix parameters such as the presence of process buffers and varying protein and nucleic acid concentrations on Virkon® S inactivation, we determined the inactivation profile of adenovirus in two different sample matrices. One sample type tested was purified adenovirus formulated in a stabilizing buffer, which contained the lowest available protein and nucleic acid concentrations. The second sample type tested was concentrated cell lysate from adenovirus-infected cells (crude virus), which contained increased concentrations of protein and nucleic acid. When compared to the purified samples, this crude virus was found to contain greater than 50-fold higher protein concentrations (as measured by a colorimetric protein quantitation assay) and greater than 100-fold higher nucleic acid concentrations (as measured by a fluorescent nucleic acid staining method). We found that the sample matrix had a significant impact on adenovirus type 5 inactivation by 0.009% Virkon® S (Fig. 1). This concentration of Virkon® S reduced purified adenovirus type 5 potency by approximately 4.5 logs in 30 min, but had no significant effect on crude adenovirus type 5 potency at the end of the same treatment time.

Multiple adenovirus type 5 constructs, prepared by replacing the adenovirus E1 gene with various HIV-1 transgenes, were evaluated for each sample matrix. No significant difference in inactivation was observed among different adenovirus type 5 constructs in the same sample matrix (Table 2). This analysis was followed by treatment of a single adenovirus type 6 construct to demonstrate the impact of varying the adenovirus serotype on Virkon® S inactivation. No significant difference in inactivation

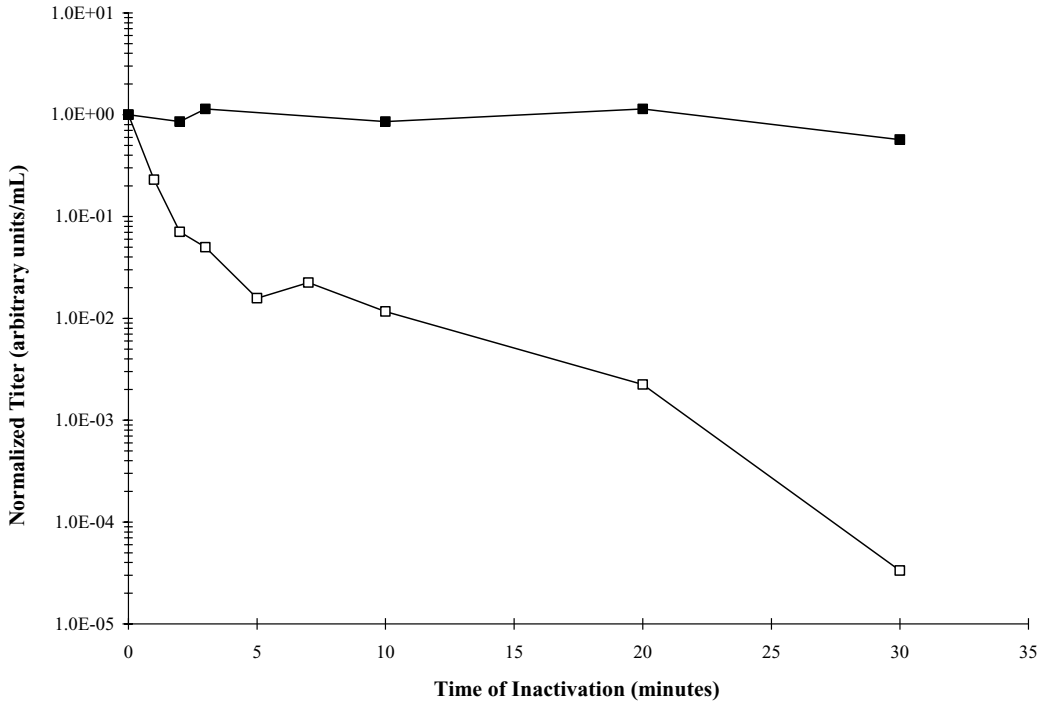

Fig. 1. Effect of organic content on the efficacy of Virkon® S inactivation. Purified (open squares) and crude (filled squares) adenovirus type 5 samples were subjected to 0.009% Virkon® S treatment, and samples were neutralized at different time points by addition of sodium thiosulfate and sodium bicarbonate. Sample potency was then assayed using the cytotoxicity-based endpoint dilution assay (see Section 2). Sample titers have been normalized to the potency of untreated virus recovered in neutralized Virkon® S.

Table 2
Log reduction values (LRV) observed after Virkon® S inactivation of various adenovirus types 5 and 6 constructs and sample matrices

Sample type	Sample description	Treatment condition	Calculated log reduction values	
			1 min exposure	5 min exposure
Adenovirus type 5	Purified, construct 1	0.09% Virkon® S	5.9	>6.5
	Purified, construct 2	0.09% Virkon® S	>6.5	>6.5
	Purified, construct 3	0.09% Virkon® S	6.8	6.2
	Unpurified, construct 1	0.9% Virkon® S	6.8	>7.3
	Unpurified, construct 2	0.9% Virkon® S	6.1	6.3
	Unpurified, construct 3	0.9% Virkon® S	5.9	6.8
Adenovirus type 6	Purified, construct 1	0.09% Virkon® S	N/A	6.7
	Unpurified, construct 1	0.9% Virkon® S	N/A	7.2

Purified adenovirus was treated with 0.09% Virkon® S, and crude adenovirus was tested at 0.9% Virkon® S, and samples were neutralized at different time points by sodium thiosulfate and sodium bicarbonate addition. Sample potency was then assayed using the cytotoxicity-based endpoint dilution assay (see Section 2). Data points marked with a "greater than" sign (>) indicate that the adenovirus titer after Virkon® S treatment fell below the assay limit of detection. Sample titers have been normalized to the potency of untreated virus recovered in neutralized Virkon® S.

was observed between the different adenovirus serotypes (Table 2).

3.3. Selecting the effective concentration of Virkon® S for adenovirus inactivation

Considering the significant impact that sample matrix has on Virkon® S efficacy against adenovirus, purified and crude adenovirus type 5 was subjected to inactivation by a wide range of Virkon® S concentrations. We found that Virkon® S concentrations at and below 0.009% were ineffective against both purified and crude adenovirus type 5 (Fig. 2). Treatment of purified adenovirus type 5 with 0.09% Virkon® S for five minutes was sufficient to reduce its infectivity to the endpoint dilution assay limit of detection. A 10-fold increase in Virkon® S concentration, 0.9%, was necessary to reduce the infectivity of crude adenovirus type 5 to the same level.

3.4. Maximum log reductions obtained using Virkon® S inactivation procedures

Based on previous results, we decided to investigate the inactivation profile of adenovirus constructs at conditions which we expected to generate the maximum log

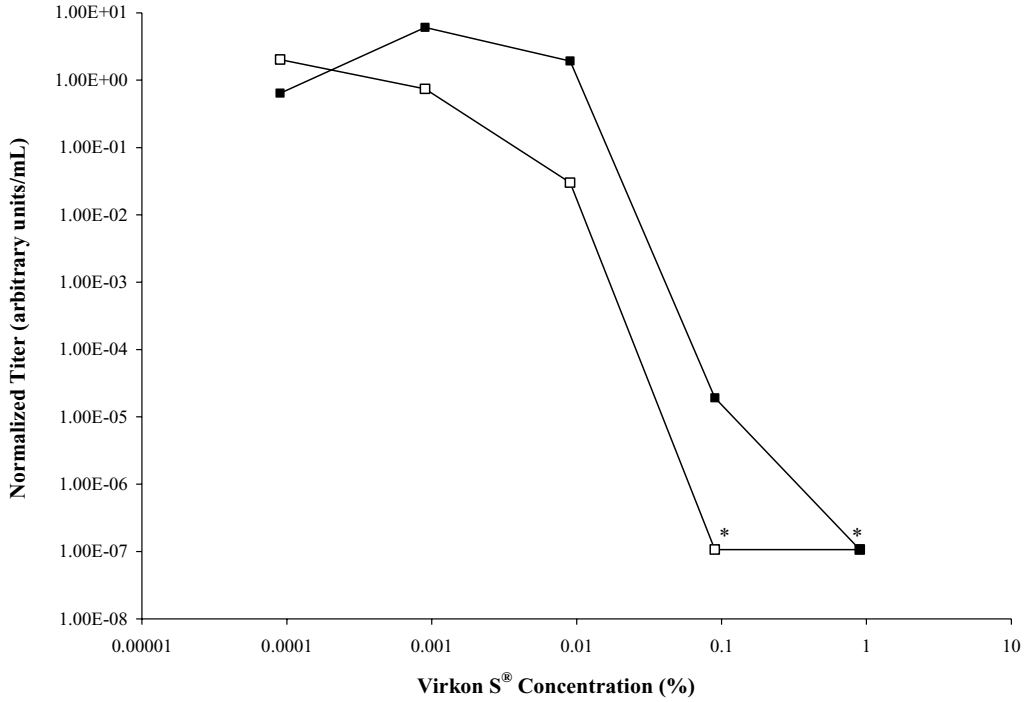

Fig. 2. Effect of Virkon® S concentration on the inactivation of purified and crude adenovirus type 5. Purified (open squares) and crude (filled squares) adenovirus type 5 samples were subjected to inactivation treatment at a range of Virkon® S concentrations for five minutes, at which time samples were neutralized by addition of sodium thiosulfate and sodium bicarbonate. Sample potency was then assayed using the cytotoxicity-based endpoint dilution assay (see Section 2). Data points marked with an asterisk (*) indicate that the purified adenovirus titer fell below the assay limit of detection at that Virkon® S concentration. Sample titers have been normalized to the potency of untreated virus recovered in neutralized Virkon® S.

reductions in a relatively short treatment time. The maximum log reduction was achieved by reducing the sample titer to below or at the endpoint dilution assay limit of detection. Purified adenovirus was treated with 0.09% Virkon® S for up to five minutes, while crude adenovirus was subjected to 0.9% Virkon® S (a 10-fold increase in concentration) for the same treatment times. The log reduction values (LRVs) in Table 2 show that purified and crude adenovirus type 5 potency was reduced by nearly six logs upon exposure to the appropriate concentration of Virkon® S for one minute, and even further after five minutes. Similar LRVs were obtained when the study was expanded to include treatment of purified and crude adenovirus type 6 with the appropriate concentration of Virkon® S for five minutes. As noted previously, the observed LRVs were independent of the type of adenovirus construct or serotype.

4. Discussion

We have described the design of an effective method for the neutralization of Virkon® S utilizing sodium thiosulfate for the neutralization of oxidative action of Virkon® S and sodium bicarbonate for the neutralization of the acidic pH of Virkon® S. We have demonstrated that the activity of Virkon® S is significantly dependent on the level of organic content in the sample matrix, as crude virus required a 10-fold increase in Virkon® S concentration compared to that used to treat purified virus to attain similar LRVs. This observation highlights the importance of considering the significant impact the presence of process buffers and varying protein and nucleic acid concentrations may have on the efficacy of chemical decontamination procedures. This issue is especially relevant for viruses, because serum and other proteins are often added to viral suspensions to protect viral infectivity from environmental conditions and to prevent degradation during freezing and storage (Gould, 1999). We also found that the LRVs obtained did not vary with the type of adenovirus construct and serotype. For future studies, it may be sufficient to generate inactivation data for one particular construct, and use that information to provide guidance and support for procedures that may be implemented for the inactivation of a different construct.

To gain confidence in the accuracy of the LRVs generated using Virkon® S to inactivate adenovirus types 5 and 6, we have incorporated appropriate control experiments to confirm the efficacy of the selected neutralization conditions, as well as to verify the absence of assay interference and cytotoxicity from the inactivation sample matrix. Using the endpoint dilution assay, we have shown that the potency of adenovirus types 5 and 6 can be reduced by greater than six logs when subjected to 0.09% Virkon® S (for purified virus) or 0.9% Virkon® S (for concentrated cell lysate) for five minutes.

Based on these results, we propose that Virkon® S liquid decontamination procedures for adenovirus types 5 and 6 use 0.9% Virkon® S for contact times greater than five minutes. Considering that the working concentration recommended by the manufacturer is 1–2%, use of Virkon® S can likely be implemented in a manufacturing setting without significant modification of process equipment or existing decontamination procedures.

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