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Evaluation of Viral Inactivation Efficacy of a Continuous Flow Ultraviolet-C Reactor (UVivatec)

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Viral safety is an important prerequisite for clinical preparations of all biopharmaceuticals derived from plasma, cell lines, or tissues of human or animal origin. To ensure the safety, implementation of multiple viral clearance (inactivation and/or removal) steps has been highly recommended for manufacturing of biopharmaceuticals. Of the possible viral clearance strategies, Ultraviolet-C (UVC) irradiation has been known as an effective viral inactivating method. However it has been dismissed by biopharmaceutical industry as a result of the potential for protein damage and the difficulty in delivering uniform doses. Recently a continuous flow UVC reactor (UVivatec) was developed to provide highly efficient mixing and maximize virus exposure to the UV light. In order to investigate the effectiveness of UVivatec to inactivate viruses without causing significant protein damage, the feasibility of the UVC irradiation process was studied with a commercial therapeutic protein. Recovery yield in the optimized condition of 3,000 J/m^2 irradiation was more than 98%. The efficacy and robustness of the UVC reactor was evaluated with regard to the inactivation of human immunodeficiency virus (HIV), hepatitis A virus (HAV), bovine herpes virus (BHV), bovine viral diarrhea virus (BVDV), porcine parvovirus (PPV), bovine parvovirus (BPV), minute virus of mice (MVM), reovirus type 3 (REO), and bovine parainfluenza virus type 3 (BPIV). Non enveloped viruses (HAV, PPV, BPV, MVM, and REO) were completely inactivated to undetectable levels by 3,000 J/m^2 irradiation. Enveloped viruses such as HIV, BVDV, and BPIV were completely inactivated to undetectable levels. However BHV was incompletely inactivated with slight residual infectivity remaining even after 3,000 J/m^2 irradiation. The log reduction factors achieved by UVC irradiation were ≥3.89 for HIV, ≥5.27 for HAV, 5.29 for BHV, ≥5.96 for BVDV, ≥4.37 for PPV, ≥3.55 for BPV, ≥3.51 for MVM, ≥4.20 for REO, and ≥4.15 for BPIV. These results indicate that UVC irradiation using UVivatec was very effective and robust in inactivating all the viruses tested.

Key words: UVC irradiation, UVivatec, virus inactivation, biopharmaceuticals, log reduction factor

The risk of virus contamination is a feature common to all biotechnology products derived from plasma, cell lines, or tissues of human or animal origin [8, 19, 26]. To ensure the viral safety, the regulatory guidelines require that manufacturers of biological products for human use must demonstrate the capability of the manufacturing process to remove or inactivate known or adventitious viruses [6, 10]. Ultraviolet-C (UVC) irradiation has been known as an effective viral inactivating method since 1940s [27]. The efficacy of UVC has also been confirmed by more recent studies [3, 9]. Therefore there have been attempts to make UV irradiation systems to inactivate viruses for biopharmaceuticals. However the systems could not handle much transmissibility, and provide low effective depth penetration, because UV irradiation intensity decreases exponentially with distance from the light source. Thus, liquid media were previously irradiated as laminar flow thin films and required prolonged exposure times to ensure virus inactivation [23].

Previous attempts to apply UV irradiation to the treatment of human serum or plasma products were not successful, with viral hepatitis being transmitted by UV-treated plasma [20, 22] and HIV by a UV-treated Factor IX concentrate [14, 15]. Recently a new generation of continuous flow UVC reactor (UVivatec) has been developed by Bayer Technology Services (Germany) to eliminate the

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need for laminar flow thin films [23, 25]. In the Bayer Technology Services UV reactor, the basic idea of the system consists of a helical channel tube formed with the semicircular outer side consisting of teflon and the straight inner side of quartz glass. The helical channel can be irradiated from the inside to the outside by a rod-shape UV light source placed inside the quartz glass. This light source is a low-pressure mercury lamp which generates UV at a specific wavelength of 254 nm. Novel hydraulic spiral flow around an irradiation source induces Dean vortices in a fluid stream. These vortices provide highly efficient mixing in a fluid stream and optimize virus exposure to the light source. As a consequence, high doses of UVC irradiation can be delivered evenly and uniformly throughout the solution. Thus, the required residence times in the irradiation chamber are extremely short and the UVC treatment can be accurately controlled.

The purpose of the present study was to examine the efficacy and robustness of UVC irradiation using UVivatec system in the inactivation of viruses in protein solutions. A variety of experimental model viruses for human pathogenic viruses, including the human immunodeficiency virus (HIV), hepatitis A virus (HAV), bovine herpes virus (BHV), bovine viral diarrhea virus (BVDV), porcine parvovirus (PPV), bovine parvovirus (BPV), minute virus of mice (MVM), reovirus type 3 (REO), and bovine parainfluenza virus type 3 (BPIV), were selected for this study (Table 1).

For the propagation and titration of HIV type 1 (HBX2 strain), BHV (ATCC VR-188), BVDV (ATCC VR-534), BPIV (ATCC VR 281), HAV (strain HM/175/18f clone B, ATCC VR-1402), PPV (ATCC VR-742), BPV (ATCC VR 767), MVM (ATCC VR 1346), and REO (ATCC VR 824), C8166 cells (European Collection of Animal Cell Culture), Madin-Derby bovine kidney (MDBK) cells (ATCC CRL-22), bovine turbinate (BT) cells (ATCC CRL-1390), Vero cells (ATCC CCL 81), FRhK-4 (ATCC CRL-1688) cells, minipig kidney (MPK) cells (ATCC CCL-166), EBTr cells (ATCC CCL 44), A9 cells (ATCC CRL 6319), and Vero C1008 cells (ATCC CRL-1586) were used, respectively as described in previous reports [4, 12, 13]. C8166 cells were grown in RPMI 1640 medium containing 2% fetal bovine serum (FBS) and L-Glutamine. Other cells were grown in high-glucose Dulbecco's modified Eagle's medium (HG DMEM) containing 2% fetal bovine serum.

An aliquot from each sample from the virus inactivation studies and an appropriate control were titrated immediately after being collected in 7-fold serial dilutions to the end point using a quantal 50% tissue culture infectious dose ($TCID_{50}$) assay [11]. For titration of HIV, suspensions of C8166 cells in 96-well culture plates were infected using at least eight 0.1 mL replicates of the appropriate dilution of sample or positive control. Negative control wells were mock-infected using at least eight 0.1 mL replicates of culture medium. The plates were incubated at 35°C for approximately 1 hr, and the wells were fed with 0.1 mL of the tissue culture medium. Approximately 14 days later the wells were examined for cytopathic effects (CPE) and syncitial formation. For titration of BHV, BVDV, BPIV, HAV, PPV, BPV, MVM, and REO, indicator cell monolayers in 24-well culture plates were infected using at least eight replicates of 0.25 mL of the appropriate dilution of each sample or the positive control. Negative control wells were mock-infected using at least eight replicates of 0.25 mL of the culture medium. The plates were then incubated at 35ºC for approximately 1 hour, and the wells were fed with 1 mL of the tissue culture medium. After 7~14 days incubation, the wells were examined for CPE.

Table 1. Salient features of viruses used for the evaluation of virus clearance.

Virus	Family	Shape	Envelop	Genome	Size (nm)	Resistance to physico-chemical reagents
Human immunodeficiency virus (HIV)	Retroviridae	Spherical	Yes	ss-RNA	120	Low
Bovine herpes virus (BHV)	Herpesviridae	Spherical	Yes	ds-DNA	130-150	medium
Bovine viral diarrhea virus (BVDV)	Flaviviridae	Spherical	Yes	ss-RNA	40-70	Medium
Hepatitis A virus (HAV)	Picornaviridae	Icosahedral	No	ss-RNA	27-32	Medium-High
Porcine parvovirus(PPV)	Parvoviridae	Icosahedral	No	ss-DNA	15-25	High
Minute virus of mice (MVM)	Parvoviridae	Icosahedral	No	ss-DNA	18-24	High
Bovine parvovirus (BPV)	Parvoviridae	Icosahedral	No	ss-DNA	18-24	High
Bovine reovirus type 3 (REO),	Reoviridae	Spherical	No	ds-RNA	60-80	Medium-High
Bovine parainfluenza virus type 3 (BPIV)	Paramyxoviridae	Pleo/Spherical	Yes	ss-RNA	100-200	Low

As a part of the virus validation protocol, cytotoxicity, interference, and load titer tests were performed, as described in previous report [24]. The cytotoxicity tests were performed on those samples generated for virus titration in the virus spiking experiments to control for any possible cytotoxic effects on the indicator cells that would interfere with the virus titration. The interference tests were performed to determine whether the starting materials for virus spiking studies exerted an inhibitory effect on the ability of the cell lines to permit the detection of the virus. The load titer assays were performed to determine precisely the point at which spiking the virus into the starting material resulted in a loss in the virus titer. The virus log reduction factor was defined as the $\log_{10}$ of the ratio of the virus loads in the spiked starting and post process materials, as described in previous report [12]. All the virus inactivation experiments were carried out in duplicate and mean values are given.

As the first step to investigate the effectiveness of UVivatec to inactivate viruses without causing significant protein damage, the feasibility of the UVC irradiation process was studied with a commercial therapeutic protein which is currently being developed by CHA Bio & Diostech Corp. (Korea). In order to optimize the UVC irradiation process, effect of UVC irradiation doses on the stability of protein solution was evaluated. The recovery yields were all more than 98% after 1,000, 2000, and 3,000 J/m^2 irradiations, respectively. In order to assure that the UVivatec UVC irradiation process will consistently operate and produce a product of the required quality, process validation study was conducted. The analysis of three batches operation with 3,000 J/m^2 irradiation showed that this UVC irradiation process has consistency and reproducibility. The physical characteristics of the product, *i.e.* color and molecular weight were not modified after UVC irradiation. No abnormal toxicity against mice was induced after UVC irradiation. The loss of protein activity was less than 2%. There was no batch to batch variation in terms of recovery yields of proteins (data not shown).

The most essential part of process validation studies for virus removal and/or inactivation is proving the validity of the scale-down process. For several reasons, that include the scale of the production process and Good Manufacturing Practice (GMP) constraints regarding the introduction of viruses, it is either impossible or impractical to perform such studies on a full manufacturing scale. Therefore, the steps to be studied have to be scale-downed to a laboratory scale [6, 10]. Table 2 shows typical comparisons of some of the process parameters for the manufacturing and scale-down runs of the UVivatec UVC irradiation process. The reliability and reproducibility of the scale-down process to perform within the production specifications were evaluated using three validation runs. A comparison of the recovery yield revealed no significant difference between the manufacturing and scale-down processes.

To evaluate the effectiveness and robustness of the UVC irradiation process in inactivating viruses, scale-down process using UVivatec Lab was challenged with viruses. Virus spiked solutions were treated with UVC with the intensities of 1,000 J/m^2, 2,000 J/m^2, and 3,000 J/m^2, respectively. According to the manufacture's manual, UVivatec was installed, and then the helical channel tube was cleaned with 2.5 L of distilled water, and disinfected with 2.5 L of 1 M NaOH. After rinsing it with phosphate buffered saline (PBS), UV lamp was turn on and warmed up to specified intensity. The total volume of the helical channel tube was 100 mL. During activation of UV lamp, 99 mL of protein solution was spiked with 11 mL of virus stock solution. After mixing, 10 mL of the virus-spiked solution was withdrawn, diluted with cell culture medium, and titrated immediately. When UV lamp was activated to the specified intensity, virus-spiked solution vessel was connected to the inlet of the helical channel tube and then inactivation mode was started. After 100 mL of virus-spiked solution run out, 100 mL of PBS solution was further passed through the helical channel tube. The fraction of virus-spiked solution was collected and then

Table 2. Comparison of some typical process parameters for UVC irradiation using UVivatec system during production runs and scale-down runs.

Item	Production runs	Scale-down runs	Scale-down factor
Working volume of test solution (mL)	10,000	100	100
Protein concentration (mg/mL)	1.5	1.5	-
Working temperature (^{o}C)	2~8	2~8	-
Working pH	7.1~7.4	7.1~7.4	-
Recovery of protein activity (%)	≥98	≥98	-

titrated immediately.

All the non-enveloped viruses tested (HAV, PPV, MVM, BPV, and REO) were completely inactivated to undetectable levels by 3,000 J/m^2 irradiation as well as 2,000 J/m^2 irradiation (Table 3). MVM was completely inactivated to undetectable levels by even 1,000 J/m^2 irradiation. The log reduction factors achieved by UVC irradiation were ≥5.27 for HAV, ≥4.37 for PPV, ≥3.51 for MVM, ≥3.55 for BPV, and ≥4.20 for REO. Also HIV, BVDV, and BPIV were completely inactivated to undetectable levels by 3,000 J/m^2 irradiation (Table 4). BVDV was completely inactivated to undetectable levels by even 1,000 J/m^2 irradiation and 2,000 J/m^2 irradiation. Although BHV was also highly sensitive to the treatment, slight residual infectivity of BHV was remaining even after 3,000 J/m^2 irradiation. BHV was inactivated from an initial titer of 8.80 $\log_{10}$ $TCID_{50}$ to 4.30 $\log_{10}$ $TCID_{50}$ by 1,000 J/m^2 irradiation. After 2,000 J/m^2 and 3,000 J/m^2 irradiation, the titer was decreased to 3.91 and 3.51 $\log_{10}$ $TCID_{50}$, respectively. The log reduction factors achieved by UVC irradiation were ≥3.89 for HIV, 5.29 for BHV, ≥5.96 for BVDV, and ≥4.15 for BPIV. These results indicate that UVC irradiation using UVivatec was very effective and robust in inactivating all the viruses tested.

The manufacture of biopharmaceuticals derived from cell lines or from human or animal plasma should be subjected to a virus clearance strategy that eliminates the risk of viral contamination common to all such products. Although biopharmaceuticals has been regarded as generally safe against viruses, which is attributed to the natural clearance effect during purification process and to the viral inactivation step such as solvent/detergent treatment, low-pH treatment, and heat treatment, a concern regarding the virus safety of this product against non-enveloped viruses such as parvovirus and HAV has been raised because of these viruses' high resistance to physicochemical treatment [2, 16]. For this reason, implementation of multiple viral clearance (inactivation and/or removal) steps has been highly recommended for manufacturing of biopharmaceuticals.

Recently, there have been a few reports describing human parvovirus B19 (B19) or HAV infections in hemophilic patients having received antihemophilic factor IX or VIII concentrate prepared from large plasma pools [5, 17, 21]. There have been also reports representing MVM contamination during the manufacture of recombinant therapeutic proteins [1, 7]. MVM belongs to the *Parvoviridae* family and is known to be highly infectious to Chinese

Table 3. Inactivation of non-enveloped viruses by UVC irradiation.

Sample	Total virus titer (Log_{10} $TCID_{50}$)				
	HAV	PPV	MVM	BPV	REO
Starting material spiked with virus	8.38	7.48	6.37	6.67	7.31
1,000 J/m^2 UVC irradiation	-[a]	-	ND(≤2.86)	-	-
2,000 J/m^2 UVC irradiation	ND[b](≤3.11)[c]	ND(≤3.11)	ND(≤2.86)	ND(≤3.12)	ND(≤3.11)
3,000 J/m^2 UVC irradiation	ND(≤3.11)	ND(≤3.11)	ND(≤2.86)	ND(≤3.12)	ND(≤3.11)
Log reduction factor	≥5.27	≥4.37	≥3.51	≥3.55	≥4.20

[a]Not determined.
[b]No infectious virus was detected.
[c]These values were calculated using a theoretical minimum detectable level of infectious virus with a 95% confidence level.

Table 4. Inactivation of enveloped viruses by UVC irradiation.

Sample	Total virus titer (Log_{10} $TCID_{50}$)			
	HIV	BHV	BVDV	BPIV
Starting material spiked with virus	7.26	8.80	9.07	7.26
1,000 J/m^2 UVC irradiation	-[a]	4.30	ND(≤3.11)	-
2,000 J/m^2 UVC irradiation	-	3.91	ND(≤3.11)	ND(≤3.11)
3,000 J/m^2 UVC irradiation	ND[b](≤3.37) [c]	3.51	ND(≤3.11)	ND(≤3.11)
Log reduction factor	≥3.89	5.29	≥5.96	≥4.15

[a]Not determined.
[b]No infectious virus was detected.
[c]These values were calculated using a theoretical minimum detectable level of infectious virus with a 95% confidence level.

hamster ovary (CHO) cells used for the production of recombinant therapeutic proteins and monoclonal antibodies [18]. Therefore, the international regulation for the validation of parvovirus and HAV safety has had to be reinforced. Consequently, the biopharmaceutical industry is paying particular attention to systems capable of removing and/or inactivating parvovirus and HAV [4, 12, 13, 16]. PPV and BPV, model viruses for B19 in viral clearance validation studies, were completely inactivated to below detectable levels by 1,000 J/m^2 irradiation. HAV was also completely inactivated to below detectable levels by 2,000 J/m^2 irradiation. MVM was also completely inactivated to below detectable levels by 1,000 J/m^2 irradiation. These results indicate that UVC irradiation using UVivatec is a robust and effective step for the elimination of non-enveloped viruses and it can be a superior alternative or supplement to common methods of virus reduction such as heat treatment, chemical disinfection, chromatography or filtration.

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국문초록

연속 유동 Ultraviolet-C 반응기(UVivatec)의 바이러스 불활화 효과 평가

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사람과 동물 유래의 혈장, 세포, 조직 등을 이용하여 생물의약품을 생산하기 위해서는 바이러스 안전성 확보가 필수적이다. 바이러스 안전성 보증을 위해 생물의약품 제조공정은 바이러스 불활화/제거 단계를 포함하여야 한다. 짧은 파장자외선(UVC) 조사는 바이러스 불활화 효과가 매우 높은 것으로 알려졌지만, UVC 조사로 인한 단백질의 변성과 대상 물질에 동일하게 조사를 할 수 있는 기계적 장치 개발의 어려움으로 인해 UVC 조사는 생물의약품 제조 공정에 사용되지 못했다. 최근에 이러한 결점을 해결한 연속 유동 UVC 반응기(UVivatec)가 개발되었다. UVivatec의 바이러스 불활화 효과 및 단백질 회수율을 검증하기 위해 단백질 의약품을 대상으로 적용가능성을 조사하였다. 최적화된 3,000 J/m^2 조사 공정에서 단백질의 회수율은 98%이상이었다. UVC 조사에 의한 human immunodeficiency virus(HIV), hepatitis A virus(HAV), bovine herpes virus(BHV), bovine viral diarrhea virus(BVDV), porcine parvovirus(PPV), bovine parvovirus(BPV), minute virus of mice(MVM), reovirus type 3(REO), bovine parainfluenza virus type 3(BPIV) 불활화 효과를 평가하였다. HAV, PPV, BPV, MVM, REO와 같은 비외피(non-envelope) 바이러스는 3,000 J/m^2 조사량에 의해 검출한계 이하로 완벽하게 불활화되었다. HIV, BVDV, BPIV 같은 외피(envelope) 바이러스도 3,000 J/m^2 조사량에 의해 검출한계 이하로 완벽하게 불활화되었다. 또한 BHV도 매우 민감하게 불활화되었다. UVC 조사에 의한 각 바이러스들의 로그 감소율은 HIV는 ≥3.89, HAV는 ≥5.27, BHV는 5.29, BVDV는 ≥5.96, PPV는 ≥4.37, BPV는 ≥3.55, MVM은 ≥3.51, REO는 ≥4.20, BPIV는 ≥4.15이었다. 이와 같은 결과에서 UVivatec을 이용한 UVC 조사는 바이러스 불활화에 매우 효과적인 방법임을 확인하였다.