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NOTE

Solvent/Detergent Inactivation and Chromatographic Removal of Human Immunodeficiency Virus During the Manufacturing of a High Purity Antihemophilic Factor VIII Concentrate

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A validation study was conducted to determine the efficacy of solvent/detergent (S/D) inactivation and Q-Sepharose column chromatographic removal of the human immunodeficiency virus (HIV) during the manufacturing of a high purity antihemophilic factor VIII (GreenMono) from human plasma. S/D treatment using the organic solvent, tri (n-butyl) phosphate, and the detergent, Triton X-100, was a robust and effective step in eliminating HIV-1. The HIV-1 titer was reduced from an initial titer of 8.3 $\log_{10}$ $TCID_{50}$ to undetectable levels within one minute of S/D treatment. HIV-1 was effectively partitioned from factor VIII during Q-Sepharose column chromatography with the log reduction factor of 4.1. These results strongly assure the safety of GreenMono from HIV.

Key words: Antihemophilic factor VIII, human immunodeficiency virus, solvent/detergent inactivation, chromatographic removal

Hemophilia A is an inherited bleeding disorder, in which the blood clotting protein factor VIII is deficient or abnormal (14). Until now hemophilia has generally been treated by the injection of coagulation factor concentrates, which are made from pooled plasma of many blood donors (3, 18).

Greencross PD Corp. is currently producing a high purity antihemophilic factor VIII (GreenMono) using monoclonal anti-FVIIIc antibodies supplied by the Hyland Division, Baxter Healthcare Corp. (USA) (9). GreenMono is manufactured from human plasma by a process that includes cryo-precipitation, column chromatography, and lyophilization, as follows. Frozen plasma is thawed at a controlled temperature, then centrifugated to isolate a factor VIII and fibrinogen-rich fraction (cryo-precipitate). The cryo-precipitate is solubilized in water for injection, then sufficient calcium chloride is added to obtain a minimum concentration of 40 μM. Subsequently the pH and temperature are adjusted to prepare a precipitate rich in fibrinogen and a supernatant rich in factor VIII. The supernatant rich in factor VIII is clarified by filtration and treated with the organic solvent tri (n-butyl) phosphate (TNBP) and the detergent Triton X-100 to inactivate lipid enveloped viruses. The final concentrations of TNBP and Triton X-100 are 0.3% (v/v) and 1.0% (v/v), respectively. The S/D treated solution (cryo/detergent solution) is clarified by filtration and applied to a monoclonal antibody (mAb) gel column to purify factor VIII. The mAb gel column eluate is applied to an ion-exchange (Q-Sepharose) column. Factor VIII is eluated from the Q-Sepharose column, and the potency is adjusted. Factor VIII is sterile-filtered prior to filling and finally lyophilized.

The production of plasma derivatives of human origin must take into account the possible presence of pathogenic viruses in the original material. The major blood-borne viruses of clinical concern include the human immunodeficiency viruses (HIV-1 and HIV-2), human T-cell leukemia virus (HTLV-1), and hepatitis B and C viruses (20). Thus, special precaution must be taken during the production of plasma-derived therapeutic products to assure against the possibility of the products transmitting infectious disease to the recipients (10, 19).

A biopharmaceutical product free from infectious viruses will in many instances not only derive solely from direct testing for their presence, but also from a demonstration that the manufacturing process is capable of removing or

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inactivating them. Validation of the process for viral inactivation/removal can play an essential and important role in establishing the safety of biological products especially when there is a high potential for the source material to be contaminated with a virus known to be pathogenic for man (8, 15, 23-25). The study presented here was thus designed to evaluate the efficacy of solvent/detergent inactivation and Q-Sepharose column chromatographic removal of HIV-1 during the manufacturing of GreenMono.

Preparation and titration of HIV-1

HIV is a member of the lentivirus family of retroviruses, which is an enveloped, medium-sized, single-stranded, RNA virus with a low resistance to physico-chemical inactivation. HIV is considered to be of concern where a product is derived from material of human origin, particularly blood products. The use of this virus is mandatory when performing a virus validation on human blood and plasma products (24, 25). Human H9 cells (ATCC HTB-176) were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and L-Glutamine. The cells were infected by mixing with H9 cells which were chronically infected with the RF strain of HIV-1 (Medical Research Council, AIDS Directed program no. 104) and the culture passaged and examined regularly for any cytopathic effect (cpe) and syncytial formation. At the peak of syncytiation, the supernatant was harvested, clarified by centrifugation, 0.45 μm filtered, aliquoted and frozen at -70°C.

An aliquot from each sample and the appropriate control were titrated immediately upon collection in 7-fold serial dilutions to the end point using a quantal 50% tissue culture infectious dose ($TCID_{50}$) assay (16). Suspensions of C8166 cells (Medical Research Council, AIDS Directed program no. 013) in microtiter 96-well plates were infected using at least eight 0.1 ml replicates of the appropriate dilution of sample or positive control HIV-1. Negative control wells were mock-infected using at least eight 0.1 ml replicates of culture medium. The plates were incubated at 37°C and the wells were fed with the tissue culture medium appropriately. Approximately 14-21 days later, the wells were examined for cpe. As a part of the virus validation protocol, cytotoxicity, interference and load titer tests were performed. Cytotoxicity tests were performed on samples generated for virus titration in virus spiking experiments to control for possible cytotoxic effects on the indicator cells which would interfere with the virus titration. Interference studies were performed to determine whether the test materials exert an inhibitory effect on the ability of the cell lines to permit detection of the virus. A load titer assay was performed to determine where spiking the virus into the starting material resulted in a loss in virus titer.

Calculation of virus reduction factor

The virus reduction factor for an individual purification or inactivation step was defined as the $\log_{10}$ of the ratio of the virus load in the spiked starting material divided by the virus load in the post process material (25). The formula took into account both the titers and volumes of the materials before and after the processing step. Reduction factors were normally expressed on a logarithmic scale.

$$10^{Ri} = (v^{I})\ (10^{aI})/(v^{II})\ (10^{aII})$$

where : Ri = reduction factor for a given stage, v^{I} = volume of the input material, aI = titer of the virus in the input material, v^{II} = volume of the retained output material, aII = titer of the virus in the output material

Validation of scale-down process

Scale-down of the purification process is an essential part in performing process validation studies for virus removal/inactivation (15, 23-25). For several reasons, including 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 these studies on the full manufacturing scale. Therefore, the steps to be studied are scaled-down to laboratory scale. The scale-down process should mimic as closely as possible the full manufacturing scale process. To ensure that the performance of the scale-down processes of S/D treatment and Q-Sepharose column chromatography were representative of those used in production, a number of validation experiments were conducted.

For the validation of the scale-down process of S/D treatment, the concentration of inactivating agents was evaluated during the virus inactivation experiments. The concentrations of TNBP and Triton X-100 were 0.29± 0.02% (v/v) and 1.02±0.05% (v/v), respectively, which were within the manufacturing specifications.

Scale-down of Q-Sepharose column chromatography was validated by comparing the column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, concentration of protein and salt, and recovery of factor VIII activity. Factor of scale-down for Q-Sepharose column chromatography was about 81. Table 1 shows a typical comparison of some process parameters for the manufacturing and scale-down processes of Q-Sepharose column chromatography. The results of five scale-down experiments demonstrated that no statistically significant difference was found between the two process scales.

Inactivation of HIV-1 during S/D treatment

Five ml of virus stock was spiked to 45 ml of Pre-S/D solution (supernatants of solubilized cryo-precipitate) equilibrated at 22°C. The virus spiked solution was treated with TNBP and Triton X-100, with the final concentrations of 0.3% (v/v) and 1.0% (v/v), respectively and then incubated at 22°C. Samples were taken at the indicated time and then 250-fold diluted with tissue culture

Table 1. Comparison of some typical process parameters for manufacturing and scale-down process of Q-Sepharose column chromatography

Item	Manufacturing Process	Scale-down Process	Scale down factor
Resin-bed dimensions	9 cm diameter 10 cm height	1 cm diameter 10 cm height	81
Volume of Q-Sepharose gel	636 ml	7.85 ml	81
Volume of mAb eluate (starting material)	14.77 L	0.18 L	81
Flow rate of elution	53.0 ml/min	0.65 ml/min	81
Average protein concentration of eluate after chromatography[a]	12.43 ± 0.10 mg/ml	12.53 ± 0.36 mg/ml	-

[a]These results were mean values of five independent experiments.

Table 2. Inactivation of HIV-1 during S/D treatment

Exp. No.	Sample	Total HIV-1 titer (Log_{10} $TCID_{50}$)
1.	Spiked starting material	8.3
	1 min after S/D treatment	ND*
	5 min after S/D treatment	ND
	30 min after S/D treatment	ND
	60 min after S/D treatment	ND
2.	Spiked starting material	8.4
	1 min after S/D treatment	ND
	5 min after S/D treatment	ND
	30 min after S/D treatment	ND
	60 min after S/D treatment	ND

*HIV-1 infectivity was not detected.

Table 3. Removal of HIV-1 during Q-Sepharose column chromatography

Exp. No.	Sample	Total HIV-1 titer (Log_{10} $TCID_{50}$)	Reduction factor (Log_{10})
1.	Spiked HIV-1	8.1	-
	mAb column eluate spiked with HIV-1	6.4	1.7
	Unbound and wash fraction	4.4	-
	Eluate fraction	2.4	4.0
	High salt wash fraction	3.4	-
2.	Spiked HIV-1	8.1	-
	mAb column eluate spiked with HIV-1	6.4	1.7
	Unbound and wash fraction	4.4	-
	Eluate fraction	2.3	4.1
	High salt wash fraction	3.4	-

medium upon collection for preventing further inactivation of viruses and cytotoxicity of S/D treated solution to indicator cells.

S/D treatment was extremely efficient in inactivating HIV-1. HIV-1 was inactivated from an initial titer of 8.3 log_{10} $TCID_{50}$ to undetectable levels within the first minute of the total 1 hour of treatment time (Table 2). From this result it was concluded that S/D treatment was a robust and effective step in eliminating HIV. Since viral load was reduced to undetectable levels within one minute of incubation in these experiments, it could be further concluded that the extent of viral inactivation was independent of the initial viral load.

There have been several reports about the viral safety of S/D treated blood products (11-13, 21). The solvent used in the S/D method is usually TNBP and the detergent is either Tween 80, Triton X-100 or sodium cholate. These reports have indicated that rapid and complete inactivation takes place in coagulation factor concentrates, immunoglobulines and whole plasma during S/D treatment. Organic solvent/detergent mixtures disrupt the membranes of viruses that have lipid envelopes. The result is either complete structural disruption or destruction of the cell receptor recognition site. In both cases the viruses are rendered non-infectious.

Removal of HIV-1 during Q-Sepharose column chromatography

To evaluate the effectiveness of the Q-Sepharose column chromatography step in eliminating HIV, the elution profile of HIV-1 during Q-Sepharose chromatography was assessed. Eluate from mAb chromatography with an elution buffer [0.05 M imidazole buffer containing 0.03 M calcium chloride, 40% polyethylene glycol (v/v), and 0.1% albumin; pH adjusted to 6.5] was spiked with HIV-1 and then a sample was immediately removed for titration. The remaining material was applied to the Q-Sepharose chromatography column equilibrated with the elution buffer for mAb chromatography. After washing the column with 20 column volumes of washing buffer [0.05 M histidine buffer containing 0.15 M sodium chloride, 1.0 mM calcium chloride, 0.1% polyethylene glycol (v/v), and 0.1% albumin; pH adjusted to 6.4], factor VIII was eluted with an elution buffer [0.05 M histidine buffer containing 0.8 M sodium chloride, 4.0 mM calcium chloride, 0.1% polyethylene glycol (v/v), and 1.0% albumin; pH adjusted to 6.0]. After elution of factor VIII, the column was washed with a high salt buffer containing 2.0 M sodium chloride to show how much HIV-1 still remained bound to the column. Unbound, wash, eluate, and high salt wash fractions were collected during the chromatography. All samples were neutralized to pH 6.5-7.5 and an aliquot of each sample was titrated immediately. The results indicate that the Q-Sepharose column was very effective in removing HIV-1, with the log reduction factor of 4.1 (Table 3). Although most of infectious HIV-1 was present in the unbound and wash fraction, HIV-1 infec-

tivity was detected in the high salt washing fraction, indicating that extensive washing and validation of the washing process are necessary for regeneration of the column. There was also a significant loss of HIV-1 titer on spiking into the starting material. The drop in titer was about 1.7 $\log_{10}$ $TCID_{50}$, indicating that eluate from mAb chromatography with an elution buffer containing 40% polyethylene glycol (v/v) exerted an inhibitory effect on the infectivity of HIV-1.

Industrial-scale chromatographic processes have been used increasingly in the last decade for plasma fractionation (1, 2, 5). Implementation and combination of ion-exchange, size-exclusion, affinity and hydrophobic interaction chromatography have allowed the development of new therapeutic products with improved purity. Also the benefit of chromatographic purification of plasma proteins in the removal of blood-borne viruses have been revealed (4, 17). There have been a few validation studies for anion-exchange chromatographic removal of HIV-1 during the manufacture of factor VIII. Validation of a process using DEAE-Fractogel TSK 650M revealed a significant log reduction factor of 3.0 (6). However, an aminohexyl-sepharose exchanger showed somewhat lower log reduction factors of 1.08 or 1.13 (7, 22). These differences may be due to a number of factors influencing the ionic or hydrophobic interactions between the virus and chromatographic material, including the characteristics of exchangers or matrices, ionic strength, pH, and composition of process buffers, or the propensity of the virus to make aggregates under certain physicochemical conditions. In addition, the overall design of each chromatographic purification step, especially the type and number of washing steps which may remove the virus from the gel prior to the elution of factor VIII, may have a significant impact on the viral reduction factor achieved.

The mAb chromatography step for GreenMono production may also be powerful means by which HIV-1 can be physically separated from factor VIII. However HIV-1 removal was not tested at this step because the process material used at this step contains S/D. The presence of S/D would effectively inactivate any spiked HIV-1 and, as a result, the effectiveness of the mAb chromatography in eliminating this virus can not be accurately determined. There are also other possible steps to inactivate or remove HIV-1 during the GreenMono process. The cryo-precipitation step can partition HIV-1 from factor VIII and also the lyophilization step can inactivate this virus. From this study it can be concluded that S/D treatment and Q-Sepharose column chromatography are powerful steps to assure the safety of GreenMono from HIV.

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