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Removal and Inactivation of Hepatitis A Virus during Manufacture of a High Purity Antihemophilic Factor VIII Concentrate from Human Plasma

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A validation study was conducted to evaluate the efficacy and mechanism of the cryo-precipitation, monoclonal anti-FVIIIc antibody (mAb) chromatography, Q-Sepharose chromatography, and lyophilization steps involved in the manufacture of high purity factor VIII (GreenMono) from human plasma, in the removal and/or inactivation of hepatitis A virus (HAV). Samples from the relevant stages of the production process were spiked with HAV and subjected to scale-down processes mimicking the manufacture of the high purity factor VIII concentrate. Samples were collected at each step and immediately titrated using a 50% tissue culture infectious dose ($TCID_{50}$) and then the virus reduction factors were evaluated. HAV was effectively partitioned from factor VIII during cryo-precipitation with the log reduction factor of 3.2. The mAb chromatography was the most effective step for removal of HAV with the log reduction factor of ≥4.3. HAV infectivity was not detected in the fraction of factor VIII, while most of HAV infectivity was recovered in the fractions of flow through and wash during mAb chromatography. Q-Sepharose chromatography showed the lowest efficacy for partitioning HAV with the log reduction factor of 0.7. Lyophilization was an effective step in inactivating HAV with the log reduction factor of 2.3. The cumulative log reduction factor, ≥10.5, achieved for the entire manufacturing process was several magnitudes greater than the potential HAV load of current plasma pools.

Key words: Antihemophilic Factor VIII, hepatitis A virus, removal, inactivation, log reduction factor

Hemophilia A is an inherited bleeding disorder, in which blood clotting protein factor VIII is deficient or abnormal (10). Until now hemophilia has generally been treated by the injection of coagulation factor concentrate, which is made from pooled plasma of many blood donors (1). Therefore special precaution must be taken during the production of this protein to assure against the possibility of the product transmitting infectious disease to the recipients (9, 17). 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 (18).

Since solvent/detergent (S/D) treatment for lipid-enveloped virus inactivation of blood products was introduced (8, 20), there have been no reported cases of such virus transmission among hemophiliacs (7, 16). However, recently there have been a few reports describing hepatitis A infections in hemophilic patients who had received factor VIII concentrate prepared from large plasma pools using a conventional anion exchange chromatography procedure coupled with S/D treatment, although it has not been clarified yet whether and how the implicated factor VIII concentrate had been contaminated with infectious hepatitis A virus (HAV) (12, 15, 19, 21, 22). A validation study for that process suggested that S/D inactivated factor VIII manufactured using anion exchange chromatography was unlikely to transmit HAV, because the combination of antibody-mediated neutralization, partitioning during cryo-precipitation and DEAE anion exchange chromatography, and inactivation during lyophilization resulted in a cumulative log HAV reduction factor of ≥8.57 (5).

Greencross PD Corp. is currently producing a high purity antihemophilic factor VIII (GreenMono) using monoclonal anti-FVIIIc antibodies (mAb) supplied by Hyland Division, Baxter Healthcare Corp. (USA) (3). The manufacturing process of GreenMono, a unique combination of process steps designed to enhance product safety, includes cryo-precipitation of pooled plasma, S/D treatment for viral inactivation, two column chromatography steps using mAb and Q-Sepharose, sterile filtration,

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filling in bottles, and lyophilization (13).

The ability to remove and/or inactivate known and potential viral contaminants during the manufacturing process of plasma derivatives has become an important parameter in assessing the safety of the products. Validation of the process for viral removal and/or inactivation can play an essential and important role in establishing the safety of plasma-derived products that have high potential for viral contamination from the source material (23-25). Accordingly, we conducted a validation study to determine the efficacy of S/D inactivation and Q-Sepharose column chromatographic removal of HIV-1 during the GreenMono process (13).

The study presented here was designed to evaluate the efficacy and mechanism of the cryo-precipitation, mAb chromatography, Q-Sepharose chromatography, and lyophilization steps involved in the manufacture of GreenMono from human plasma, in the removal and/or inactivation of HAV.

Materials and Methods

Validation of process scale-down

Scale-down of the purification process is an essential part in performing process validation studies for virus removal/inactivation (23-25). For several reasons, including the scale of the production process and Good Manufacturing Practice (GMP) constraints regarding 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 cryo-precipitation, column chromatography and lyophilization were representative of those used in production, a number of validation experiments were conducted.

To ensure that the scale-down process of cryo-precipitation was representative of production scale, physicochemical properties such as pH, temperature, concentrations of proteins and salts, and the recovery of factor VIII activity in cryo-precipitate were compared.

Scale-down of mAb and Q-Sepharose column chromatography were 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 proteins and salts, and recovery of factor VIII activity. Factors of scale-down for mAb and Q-Sepharose column chromatography were about 623 and 81, respectively.

For the comparison of the lyophilization process, moisture content, solubility and activity of factor VIII concentrates after freeze-drying were evaluated. Lyophilization was performed using an experimental freeze dryer (VirTis, Genesis 25XL, USA)

Preparation of HAV stock

FRhK-4 (ATCC CRL-1688) cells were grown in high glucose Dulbeccos modified Eagles medium (HG DMEM) containing 2% fetal bovine serum (FBS). Cell monolayers were infected with HAV strain HM/175/18f clone B (ATCC VR-1402) and the culture was examined regularly for cytopathic effect (cpe). When cpe was evident, the culture supernatant and cell debris were frozen and thawed once and then harvested. The cell debris was removed by centrifugation and the resultant supernatant was 0.45 μm filtered, aliquoted and frozen at -70°C. All the experiments including preparation and titration of HAV and virus spiking experiments were performed in a Category III containment laboratory.

Titration of HAV

An aliquot from each sample and the appropriate control were titrated immediately upon collection in 7-fold serial dilutions to end point in a quantal 50% tissue culture infectious dose ($TCID_{50}$) assay using FRhK-4 (ATCC CRL-1688) cells. Cell monolayers in 24-well culture plates were infected using at least eight 0.25 ml replicates of the appropriate dilution of sample or positive control. Negative control wells were mock-infected using at least eight 0.25 ml replicates of culture medium. The plates were incubated at 37°C for approximately 1 hour, and the wells were fed with 1 ml of tissue culture medium.

Test of cytotoxicity and interference

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. All samples were provided from experiments that had been in the control process under the scale-down or full manufacturing scale conditions without viral spikes. All samples, where necessary, were pH adjusted to pH 6.5 to 7.5 and 0.45 μm filtered. Each sample was tested for cytotoxicity at full strength and also at an appropriate range of doubling dilutions. Interference studies were performed to determine whether the cryo-precipitate and the product fractions from the chromatography steps exerted an inhibitory effect on the ability of the cell lines to permit detection of the virus. Aliquots of the virus were spiked into doubling dilutions of neutralized samples to give final concentrations within the range 10^0 to 10^3 infectious units. The dilutions were then assayed for the presence of infectious virus with two replicates per virus concentration per buffer dilution tested. A positive control for the virus was titrated concomitantly with these samples : HAV was spiked into tissue culture medium and diluted with tissue culture medium and then assayed for infectious virus.

Load titer assay

To determine whether there was a loss of HAV titer on spiking into the starting material, an independent aliquot of starting material was spiked with HAV and titrated in serial 7-fold dilutions through tissue culture medium. A positive control for the virus was titrated concomitantly with these samples: HAV was spiked into tissue culture medium and diluted with tissue culture medium and then assayed for infectious virus.

Virus spiking studies

Process validation experiments were performed by spiking the starting material for each step of the process with an aliquot of HAV stock solution in a volume that was 10% (v/v) of the total volume of the material. This was to ensure that the nature of the starting material was not affected by adding virus in tissue culture medium. A control sample was taken after the addition of HAV to the starting material. After subjecting the sample to the processing step, the appropriate fractions were collected for assay of infectious virus.

The first step in factor VIII manufacture is separation of the cryo-precipitate from other plasma proteins. To determine how HAV partitions during this process, 5 ml of HAV stock was spiked to 45 ml of three different pooled plasma samples which had been thawed at 4°C. The cryo-precipitate was collected by slow speed centrifugation ($4000 \times g$, 3 min), and the virus content of the supernatant and resuspended cryo-precipitate fractions were compared with that of spiked-pooled plasma.

To determine how HAV partitions during mAb column chromatography, the supernatant rich in factor VIII after cryo-precipitation was spiked with HAV and then a sample was immediately removed for HAV titration. The remaining spiked solution was treated with S/D and a post S/D treated sample was removed for HAV titration. The remaining material was applied to the mAb chromatography column to which factor VIII specifically binds. The mAb column was extensively washed to remove unbound protein contaminants, virus, and S/D mixture. After washing the column with 0.05 M imidazole buffer containing 5% ethylene glycol (pH 6.4), factor VIII was eluted with an elution buffer containing 0.05 M imidazole, 40% ethylene glycol, and 1% albumin (pH 6.5). After elution of factor VIII, the column was washed with a high salt buffer containing 2.0 M sodium chloride to show how much HAV still remained bound to the column. The unbound, wash, eluate, and high salt wash fractions were collected. All samples were neutralized to pH 6.5-7.5 and an aliquot of each sample was titrated immediately.

To evaluate the effectiveness of the Q-Sepharose column chromatography step in eliminating HAV, the elution profile of HAV during Q-Sepharose chromatography was assessed as described before (13).

Calculation of virus reduction factors

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 as previously described (13).

Quantification of total antibody to HAV

The titer of anti-HAV antibody in plasma pools was determined using a commercially available microparticle enzyme immunoassay (HAVAB® 2.0 Quantitative, Abbott Laboratories).

Nested polymerase chain reaction (PCR) analysis of HAV

15 batches of GreenMono were tested for the presence of HAV gene sequences by a nested PCR according to Grinde *et al.* (4). The primers were: HAV6, 5'-TGTCTGGAGCACTGGATGG-3' (2839-2857); HAV7, 5'-CATTTCAAGAGTCCACACACTTCT-3' (3357-3380); HAV8, 5'-TGGTTTCTATTCAGATTGCAAATTA-3' (2890-2914); and HAV9, 5'-TTCATTATTTCATGCTCCTCAGT-3' (3264-3286). The numbers refer to the HM/175 strain.

Results

Validation of scale-down process

The parameters of the scale-down process of cryo-precipitation were compared with those of the manufacturing process. All the parameters obtained for the scale-down process were within the manufacturing specifications. Recovery of cryo-paste from pooled plasma during the scale-down and manufacturing processes were 1.33±0.05 mg/ml and 1.31 ± 0.12 mg/ml, respectively.

Table 1 shows a typical comparison of some process parameters for the manufacturing and scale-down processes of mAb column chromatography. The results of five scale-down experiments demonstrated that no statistically significant difference was found between factor VIII recovery for the two process scales.

The results of comparison of some typical process parameters for the manufacturing and scale-down processes of Q-Sepharose chromatography have already been described in a previous report (13), in which it was demonstrated that no statistically significant difference was found between the two process scales.

For the comparison of the lyophilization process, key product quality measurements such as moisture content, solubility and recovery of factor VIII activity after freeze-drying were evaluated (Table 2). The results indicate that the lyophilization conditions were within the manufacturing specifications.

Partitioning of HAV during cryo-precipitation

The partitioning profile of HAV after cryo-precipitation

Table 1. Comparison of some typical process parameters for manufacturing and scale-down processes of mAb gel column chromatography

Item	Manufacturing Process	Scale-down Process	Scale down factor
Resin-bed dimensions	25 cm diameter 9.4 cm height	1.0 cm diameter 9.4 cm height	623
Volume of mAb gel	4612 ml	7.4 ml	623
Volume of cryo-detergent solution	187 L	0.3 L	623
Flow rate of elution	155.8 ml/min	0.25 ml/min	623
Average percent recovery[a]	83 ± 6%	87 ± 5%	-

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

Table 2. Comparison of lyophilization performance achieved during the manufacturing and scale-down processes[a]

Process	Solubility at room temp. (second)	Moisture (%)	Recovery of factor VIII activity after lyophilization (%)
Manufacturing process	11.3 ± 4.0	1.20 ± 0.3	97.3 ± 0.7
Scale-down process	10.7 ± 4.0	1.13 ± 0.04	97.1 ± 0.6

[a]These results are mean values of three independent experiments.

Table 3. Partitioning of HAV during cryo-precipitation[a]

Sample	Total HAV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Plasma spiked with HAV	6.9 ± 0.2	-
Cryo-precipitate	3.7 ± 0.1	3.2
Supernatant fraction	6.8 ± 0.2	-

[a]These results are mean values of three independent experiments.

Table 4. Removal of HAV during mAb column chromatography[a]

Sample	Total HAV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Cryo-precipitate spiked with HAV	7.5 ± 0.4	-
Post solvent/detergent treatment	6.3 ± 0.2	-
Unbound fraction	5.8 ± 0.3	-
Wash fraction	4.6 ± 0.5	-
Eluate fraction	ND[b] (3.2[c])	≥4.3
High salt wash fraction	ND (3.2)	-

[a]These results are mean values of three independent experiments.
[b]HAV infectivity was not detected.
[c]Theoretical minimum detectable levels were used for calculation where HAV infectivity was not detected.

Table 5. Removal of HAV during Q-Sepharose column chromatography[a]

Sample	Total HAV titer (Log_{10} $TCID_{50}$)	Log reduction factor
mAb column eluate spiked with HAV	8.4 ± 0.3	-
Unbound and wash fraction	5.7 ± 0.2	-
Eluate fraction	7.7 ± 0.3	0.7
High salt wash fraction	6.9 ± 0.2	-

[a]These results are mean values of three independent experiments.

was assessed in order to evaluate the efficacy of the process in eliminating HAV (Table 3). Most of the infectious virus was detected in the supernatant fraction, while only a little HAV was present in the cryo-precipitate, thereby indicating that HAV was effectively partitioned from factor VIII. The log reduction factor achieved was 3.2.

Removal of HAV during mAb column chromatography

To evaluate the effectiveness of the immunoaffinity column chromatography step in eliminating HAV, the elution profile of HAV during mAb chromatography was assessed (Table 4). No infectious virus was detected in the eluate fraction and high salt wash fraction, thereby indicating the complete removal of HAV during mAb chromatography. The average log reduction factor achieved was $\geq$ 4.3.

Removal of HAV during Q-Sepharose column chromatography

To evaluate the effectiveness of the Q-Sepharose column chromatography step in eliminating HAV, the elution profile of HAV during Q-Sepharose chromatography was assessed (Table 5). The results indicate that the Q-Sepharose column was not effective for removing HAV. The log reduction factor was less than 1.

Inactivation of HAV during lyophilization

The final step in the manufacture of factor VIII is freeze-drying. HAV stock was spiked to the final factor VIII concentrate solution and then the HAV-spiked samples were distributed in final containers. The titer of HAV was measured before and after lyophilization. The results in Table 6 indicate that HAV was potentially sensitive to the lyophilization process, with an average log reduction factor of 2.3.

HAV PCR analysis of GreenMono

GreenMono products were examined for the presence of HAV RNA sequences by a nested PCR. An example of HAV PCR analysis is shown in Fig. 1. As the positive controls, 25 fold dilutions of HAV positive sera with HAV negative sera, 125 or 1,250 fold dilutions of HAV positive sera with GreenMono product solution, and 100 fold dilutions of the cultured HAV strain HM/175/18f clone B

Table 6. Inactivation of HAV during lyophilization[a]

Sample	Total HAV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Before lyophilization	7.1 ± 0.5	-
After lyophilization	4.8 ± 0.1	2.3

[a]These results are mean values of three independent experiments.

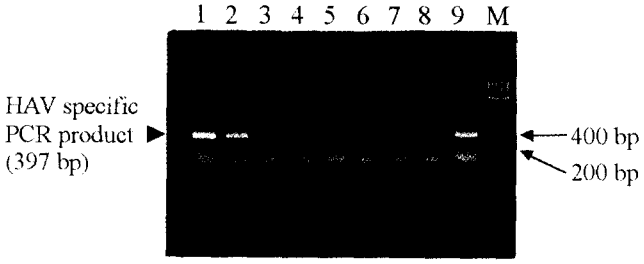

Fig. 1. Detection of HAV RNA by nested PCR. Amplified products were applied to an agarose gel. Lanes: 1, 25 fold dilutions of HAV positive sera with HAV negative sera; 2, 125 fold dilutions of HAV positive sera with GreenMono product solution (Lot No.370A0002); 3, 1,250 fold dilutions of HAV positive sera with GreenMono product solution (Lot No. 370A0002); 4, GreenMono product (Lot No. 370A0002); 5, GreenMono product (Lot No. 370A0006); 6, GreenMono product (Lot No. 370A0008); 7, GreenMono product (Lot No. 370A0010); 8, GreenMono product (Lot No. 370A0012); 9, 100 fold dilutions of the cultured HAV strain HM/175/18f clone B with GreenMono product solution (Lot No. 370A0002); M, molecular marker (200 bp DNA ladder).

with GreenMono product solution were used. All the positive controls showed a typical HAV specific PCR product, but all the purified factor VIII concentrates tested were negative for HAV PCR.

Discussion

The viral safety of plasma-derived antihemophilic factors is of major concern in the treatment of hemophiliacs. Approaches to improve the safety of plasma-derived products are based on four principles : a) careful selection of donors; b) careful screening of donated units for known infectious agents; c) the use of validated manufacturing methods, which include specific steps designed to remove or inactivate viruses; and d) testing of products and intermediates for viral markers. According to these principles, GreenMono, a high purity antihemophilic factor VIII, is manufactured from plasma pools which are negative for the hepatitis B surface antigen, anti-HIV-1-anibody, anti-HCV-antibody, and hepatitis A RNA based on PCR. Recently, HAV safety has been a hot issue for hemophiliacs who are treated with factor VIII derived from plasma. HAV is a member of the *Picornaviridae* family, which are non-enveloped, small (25-30 nm), single-stranded RNA viruses with a medium to high resistance to physico-chemical inactivation (2). In this study, the fate of HAV during several key steps in the manufacture of GreenMono was studied in order to obtain a comprehensive understanding of removal and/or inactivation of HAV through the process.

The great majority of HAV was effectively partitioned from factor VIII, as indicated by the log reduction factor of 3.2 obtained during the cryo-precipitation process (Table 3). This process has also been reported to be highly effective in partitioning HAV with a log reduction factor of 2.63 (5).

Although S/D treatment was extremely efficient in inactivating HIV-1 (13), it was not effective against HAV. The difference of total HAV titer before and after S/D treatment of cryo-precipitate was 1.2 $\log_{10}$ $TCID_{50}$ (Table 4).

The immunoaffinity column chromatography using anti-human factor VIII : C monoclonal antibody matrices was the most effective step for removal of HAV from the factor VIII concentrates (Table 4). The log reduction factor for HAV was $\geq$ 4.10. During the mAb column chromatography process, factor VIII specifically bound to the monoclonal antibodies, while the contaminants including HAV, S/D, and residual proteins were removed by extensive washing. Notably, most of the HAV did not bind to the mAb column, but flowed through the column. The inclusion of an inactivation or removal step reducing titers of the relevant viruses by a factor of 4 $\log_{10}$ or more has been recommended for the manufacturing of plasma products (11, 23). In this regard, this mAb process is a critical step to ensure the safety of factor VIII concentrate.

The Q-Sepharose column chromatography, an ion-exchange step in the manufacture of factor VIII concentrates used to further reduce impurities was found to be minimally effective against HAV (Table 5). The log reduction factor was <1.0. Another anion-exchange chromatography process using a DEAE column has also been reported to be less effective for removal of HAV, with the log reduction value of 1.27 (5, 14).

The loss of 2.3 $\log_{10}$ ($TCID_{50}$) of HAV infectivity during the freeze-drying process (Table 6) agrees well with the previous observations of Hamman *et al.* (5) and Hart *et al.* (6) who reported log reduction factors of 2.28, and 2.0, respectively from the manufacturing process for factor VIII using an anion-exchange column chromatography. The mechanism by which HAV is inactivated as a result of lyophilization is poorly understood, but probably results in destabilisation of quaternary interactions between components of the capsid. Under normal conditions, water of solvation will be important in maintaining protein secondary structure and may also be involved in hydrogen bond formation between different sub-units. This water would be removed during the lyophilisation process, which could result in disruption of normal protein interactions.

The cumulative virus reduction factor for a manufacturing process is determined from the sum of the indi-

vidual virus reduction factors based on individual process steps of different physicochemical methods (25). However, reduction in the virus titer of the order of 1 $\log_{10}$ or less would be considered negligible and would be ignored unless otherwise justified. Although the log reduction factor for Q-Sepharose column chromatography was 0.7, this value was reproducible. Therefore the cumulative log HAV reduction factor achieved through different process steps in the manufacture of the antihemophilic factor VIII was ≥10.5, which was several magnitudes greater than the potential virus load of current plasma pools. The results of these experiments showed that the GreenMono process strongly assures the safety of GreenMono from HAV.

Neutralization of HAV by HAV antibody in plasma pools is a possible mechanism of HAV removal during the GreenMono process. The neutralization of HAV by HAV antibody in plasma pools has been revealed (5, 14). The average anti-HAV titer in plasma pools from New York Blood Center donors was 1.75 IU/ml and the neutralizing effect of the plasma pools was significant with the log reduction factor of ≥ 2.15. The anti-HAV titers in plasma pools from Korea Red Cross donors used for manufacture of GreenMono ranged from 1.86 to 2.88 IU/ml, which were higher than those from New York Blood Center donors.

All the plasma for the manufacture of GreenMono is tested for the presence of HAV gene sequences using a nested PCR and then only PCR-negative plasma is used for the manufacture of factor VIII. All the final products are also tested for the presence of HAV gene sequences. Over 15 batches of GreenMono were examined for HAV gene sequences by PCR. All were negative (Fig. 1). These PCR data support the effectiveness of the GreenMono process for HAV removal demonstrated by HAV-spiking experiments.

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