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Enhanced Virus Safety of a Solvent/Detergent-Treated Antihemophilic Factor IX Concentrate by Dry-Heat Treatment

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Abstract With particular regards to the hepatitis A virus (HAV), a terminal dry-heat treatment (100°C for 30 min) process, following lyophilization, was developed to improve the virus safety of a solvent/detergent-treated antihemophilic factor IX concentrate. The loss of factor IX activity during dry-heat treatment was of about 3%, as estimated by a clotting assay. No substantial changes were observed in the physical and biochemical characteristics of the dry-heat-treated factor IX compared with those of the factor IX before dry-heat treatment. The dry-heat-treated factor IX was stable for up to 24 months at 4°C. The dry-heat treatment after lyophilization was an effective process for inactivating viruses. The HAV and murine encephalomyocarditis virus (EMCV) were completely inactivated to below detectable levels within 10 min of the dry-heat treatment. Porcine parvovirus (PPV) and bovine herpes virus (BHV) were potentially sensitive to the treatment. The log reduction factors achieved during lyophilization and dry-heat treatment were ≥5.60 for HAV, ≥6.08 for EMCV, 2.64 for PPV, and 3.59 for BHV. These results indicate that dry-heat treatment improves the virus safety of factor IX concentrates, without destroying the activity. Moreover, the treatment represents an effective measure for the inactivation of non-lipid enveloped viruses, in particular HAV, which is resistant to solvent/detergent treatment.

Keywords: antihemophilic factor IX, dry-heat treatment, hepatitis A virus, virus inactivation

INTRODUCTION

Hemophilia B is an inherited bleeding disorder, where the blood clotting factor IX is deficient or abnormal. Until now, hemophilia has generally been treated by the injection of coagulation factor concentrates made from the pooled plasma of many blood donors [1]. The use of biopharmaceutical products derived from human plasma has previously been associated with the frequent transmission of human immunodeficiency virus type 1 (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV). The development of virus inactivation and/or removal technologies has greatly reduced the frequency of such transmissions [2-4]. Since the advent of solvent/detergent (S/D) treatment for plasma-derived products, there has been no reported transmission of enveloped viruses such as HIV, HBV, or HCV, by treated products [5,6]. Although S/D treated blood products have become widely available over the last 15 years, this treatment generally has no effect on non-enveloped viruses. Consequently, the blood industry is paying particular attention to systems capable of removing and/or inactivating non-enveloped viruses, such as hepatitis A virus (HAV) and human parvovirus B19 [7-9].

Recently, there have been a few reports describing HAV infections in hemophilic patients having received antihemophilic factor IX or VIII concentrate prepared from large plasma pools using a conventional anion exchange chromatography procedure coupled with S/D treatment, although whether and how the implicated antihemophilic factor concentrates became contaminated with infectious HAV remain to be clarified [10-12]. Therefore, the international regulation for the validation of HAV safety has had to be reinforced. 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 [13].

The Green Cross Corp. is currently producing an antihemophilic factor IX complex (FacNine) using conventional ion-exchange chromatography. The manufacturing process of FacNine includes S/D treatment for viral inactivation. Therefore, there had been a great need for the implementation of an orthogonal method for inactivating non-enveloped viruses, with particular regards to HAV, for improving the safety of the factor IX.

The integration of a virucidal method has varying consequences for the general design of a manufacturing process. For pasteurization, high concentrations of stabilizers are needed during heating in the liquid state in or-

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der to ensure survival of the labile protein, which subsequently have to be removed from the products [14]. However, dry-heat treatment after lyophilization of the product can be performed with no stabilizers addition. Therefore, dry-heat treatment is one of the choices generally recommended for virus inactivation [15-17].

The aim of this study was to improve the safety of factor IX concentrate, with regards to non-lipid enveloped viruses. For this purpose, the optimal dry-heat conditions were determined, and the virucidal efficacy of the treatment was investigated using three non-enveloped viruses; HAV, murine encephalomyocarditis virus (EMCV; a model virus for hepatitis A virus), porcine parvovirus (PPV; a model virus for human parvovirus), and an enveloped virus; bovine herpes virus (BHV; a model virus for the human herpesvirus, such as HHV-6, HHV-7, HHV-8, Epstein Barr virus, or HSV-1).

MATERIALS AND METHODS

Preparation of Factor IX Solution

The factor IX concentrate used in this study was a factor IX complex prepared by conventional ion exchange chromatography, with a solvent-detergent step for virus inactivation. The factor IX complex was commercialized by Green Cross Corp., under the name FacNine. Factor IX solution was obtained from the production batches of the Green Cross Corp., and stored below −70°C until use. The potency of the solution was about 30 IU of factor IX per milliliter. One IU of factor IX is the amount contained in one mL of normal human plasma.

Biological, Physical, and Chemical Analysis

All the analyses were performed according to Standard Operating Procedure (SOP), based on the Korean Pharmacopoeia, British Pharmacopoeia and US Pharmacopoeia. The factor IX activity was determined using the clotting method with factor IX deficient plasma. Clotting times were determined on a KC10 coagulometer (Amelung, Lemgo, Germany). The residual moisture content of the lyophilized factor IX was determined by a loss on drying method. Abnormal toxicity was determined by the abdominal injection of a test sample into mice, with the subsequent monitoring of the toxicity and skin reactivity for up to 7 days post treatment. The presence of pyrogens was detected by administration of the test sample to the ear vein of rabbits, with subsequent monitoring for temperature changes.

Effect of Lyophilization on Factor IX Activity

The effect of lyophilization on the activity of factor IX was evaluated either with or without the addition of stabilizing agents. The stabilizing agents were 10 mM sodium citrate, 120 mM glycine and 220 mM sodium chloride. Factor IX solution was distributed in final containers at 10 mL/vial. The vials were loaded into a freeze dryer (Genesis 25XL, VirTis, NY, USA), which had been precooled to −40°C, and held for 4 h for pre-freezing. After switching the vacuum on, the temperature was maintained at −40°C for 1 h for stabilization. The temperature was then ramped to −10°C at 10°C/h, and held at this temperature for 33 h. The temperature was then ramped to 19°C at 5°C/h, and held at this temperature for a further 12 h. The vials of freeze dried factor IX were closed under vacuum and capped before storage at 4°C. The factor IX activities, both before and after lyophilization, were measured and the residual moisture content of lyophilized factor IX determined.

Effect of Dry-Heat Treatment on Factor IX Activity

The effect of dry-heat treatment on the activity of factor IX was evaluated. The lyophilized final products were loaded into a convection drying oven (Korea Science, Seoul, Korea), equilibrated at 30°C. Dry-heat treatment was then processed at 60 ± 1, 80 ± 1, and 100 ± 1°C, respectively. The temperature was continuously monitored in several reference bottles, and recorded using a Temperature Validator (Korea Science). As soon as the temperature reached that specified, the incubation period of dry-heating was initiated. Samples were taken at the indicated times and the factor IX activity of the dry-heated products measured.

Virus Inactivation Studies

There is a requirement to show that all viral clearance processes are effective under worst-case conditions. Therefore, it has been recommended that a virus validation study should be performed under worst-case conditions to demonstrate the minimum clearance that can be provided by an individual step [18]. As the worst-case condition for dry-heat treatment of 30 min at 100 ± 1°C, 99 ± 1°C was adopted. The virus stock was spiked to the final factor IX complex solution as 10% (v/v) of the total volume of the material. The virus-spiked samples were distributed in final containers at 10 mL/vial. As reference samples for monitoring the temperature, factor IX activity and residual moisture content, the factor IX solution, without the addition of virus, was filled into the vials. After lyophilization, the residual moisture content of the freeze dried factor IX was determined. Titers of viruses were measured before and after lyophilization. The lyophilized samples were loaded into a convection drying oven (Korea Science), equilibrated at 30°C. The dry heat-treatment then processed for 30 min at 99 ± 1°C. As soon as the temperature reached 99°C, the incubation period of dry-heating was initiated. Samples were removed at different times, and the titers of the viruses then measured.

Preparation and Titration of Viruses

The virucidal efficacy of the dry-heat treatment was investigated using four different model and relevant viruses; HAV strain HM/175/18f clone B (ATCC VR-

1402), EMCV (ATCC VR-129B), PPV (ATCC VR-742), and BHV (ATCC VR-188). For the propagation and titration of HAV, EMCV, PPV, and BHV, FRhK-4 (ATCC CRL-1688), Vero cells (ATCC CCL-81), Minipig kidney (MPK) cells (ATCC CCL-166), and Madin-Derby bovine kidney (MDBK) cells (ATCC CRL-22) were respectively used, as described in previous reports [19,20]. All the cells were grown in a 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 [21]. Indicator cell monolayers in 24-well culture plates were then 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 35°C for approximately 1 h, and the wells were fed with 1 mL of the tissue culture medium. After 7~14 days incubation, the wells were examined for cytopathic effect.

As a part of the virus validation protocol, cytotoxicity, interference, and load titer tests were also performed. The cytotoxicity tests were performed on those samples generated for virus titration in the virus spiking experiments to control any possible cytotoxic effects on the indicator cells which would interfere with the virus titration. Indicator cell monolayers in 24-well culture plates were infected using at least eight replicates of 0.25 mL of a range of doubling dilutions of each sample. The plates were then incubated at 35°C for approximately 1 h, and the wells were fed with 1 mL of the tissue culture medium. After appropriate incubation, the cytotoxic effect of each sample was measured. 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. Aliquots of virus were spiked into doubling dilutions of sample to give final concentrations within the range of 10^0 to 10^5 infectious units. The dilutions were then assayed for the presence of infectious virus with two replicates per virus concentration per buffer dilution tested. 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.

Calculation of Virus Reduction Factors and Statistical Methods

The virus 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 [22]. All the virus inactivation experiments were carried out three times. Mean values and standard deviations of three independent experiments were evaluated using Microsoft Excel program.

Table 1. Factor IX activity before and after lyophilization

Sample	Factor IX (IU/mL)
Before lyophilization	30.2 ± 1.4
After lyophilization	
Without stabilizing agents	20.4 ± 2.5
With stabilizing agents	29.4 ± 1.8

These results are the mean values of ten independent experiments.

RESULTS AND DISCUSSION

Factor IX Activity and Residual Moisture after Lyophilization

The instability of proteins is found to be one of the major constrains in the development of biopharmaceuticals [23]. Lyophilization is a standard method for stabilizing labile products that have limited shelf-lives when in a dilute solution. Lyophilization may be defined as the drying of a substance by its freezing, with the removal of a portion of any associated solvent by sublimination directly from the solid to the gaseous phase, without passing through the intermediate liquid phase. The stability of a protein during lyophilization can be influenced by the excipients [24]. Without appropriate stabilizing excipients, most protein preparations are at least partially denatured due to the freezing and dehydration stresses encountered during lyophilization. Therefore, stabilizers, such as amino acids, citrate or sugars, should be added in order to maintain the biological function of heat labile proteins. From a study on the effect of various classes of excipients on the stability of factor IX during lyophilization, 10 mM sodium citrate, 120 mM glycine, and 220 mM sodium chloride were chosen as stabilizing agents. The factor IX activities, both before and after lyophilization, were measured (Table 1). The loss of factor IX activity in the absence of stabilizing agents was more than 30% after lyophilization. However, a slight drop in the factor IX activity, within the range of 2~3%, was observed in the presence of the excipients. This small loss of factor IX activity during lyophilization was clearly due to the addition of the stabilizing excipients to the factor IX solution.

The moisture content of lyophilized products affects the stability. The residual moisture content after lyophilization should be within the limits that no longer support chemical reactions or biological growth. A low moisture content, typically below 2%, results in good storage stability [25]. Korean Pharmacopoeia has recommended that the moisture content of lyophilized biopharmaceuticals, including antihemophilic factor IX, should be below 3%. Therefore, the moisture contents of the lyophilized products were measured. The mean (N = 10) residual moisture value in lyophilized factor IX was 0.77%.

Effect of Dry-Heat Conditions on the Activity of Factor IX

There have been several reports on dry-heat treatment

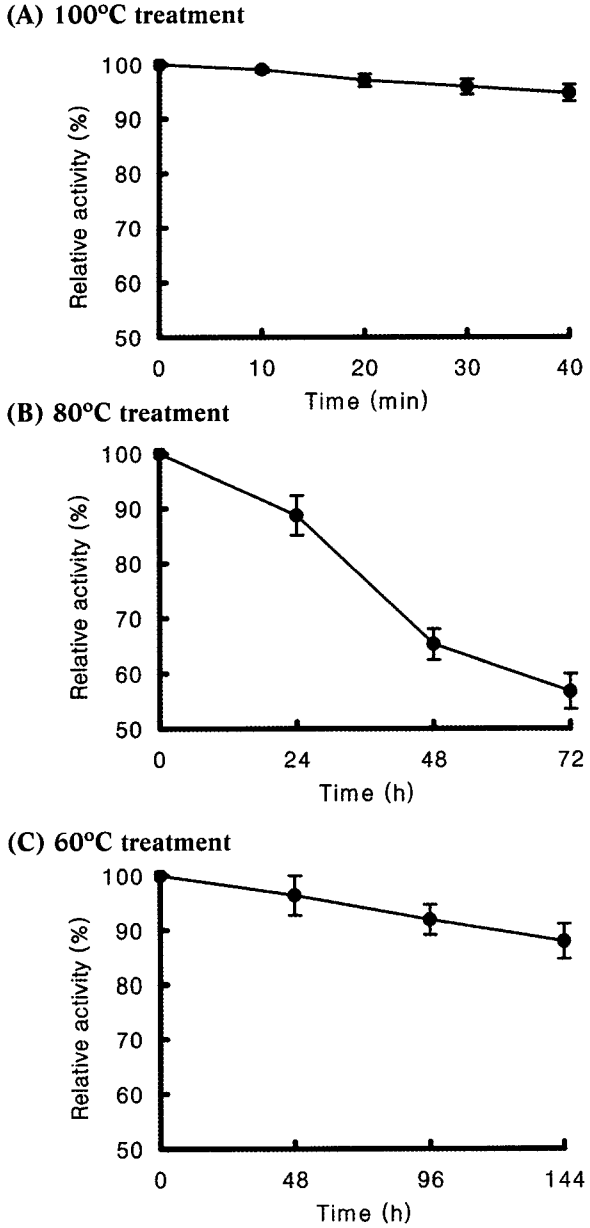

Fig. 1. Effect of dry-heat temperature on the activity of antihemophilic factor IX. These results are the mean values of three independent experiments.

for improving the virus safety of antihemophilic factor VIII [15-17,26]. However, less has been reported about factor IX. Dry-heat treatments at 100°C for 30 min, 80°C for 72 h, or 60°C for 144 h have been generally recommended for improving the virus safety of factor VIII. Therefore, the effect of the dry-heat temperature, at 100, 80, and 60°C, was examined on the activity of the lyophilized factor IX as a function of time (Fig. 1). The activity of the factor IX decreased during dry-heat treatment. The loss of factor IX activity during the treatment at 100 ± 1°C for 30 min was 5%. However, the losses during the treatments at 80 ± 1°C for 72 h and 60 ± 1°C for 144 h were about 43 and 12%, respectively. Therefore, dry-heat treatment at 100 ± 1°C for 30 min was chosen as the viral inactivation process for improving the safety of factor IX.

Table 2. Characteristics of factor IX products before and after dry-heat treatment

Items	Before treatment	After treatment
Activity (IU/vial)	291.93 ± 4.70	276.87 ± 5.67
Moisture content (%)	0.77 ± 0.31	0.78 ± 0.38
Total protein (mg/mL)	6.20 ± 1.13	6.17 ± 1.16
pH	7.03 ± 0.11	7.04 ± 0.10
Solubility (min)	0.70 ± 0.00	0.70 ± 0.00
Non soluble materials	Negative	Negative
Pyrogens (°C)	0.72 ± 0.18	0.61 ± 0.25
Abnormal toxicity against mouse	Negative	Negative

These results are the mean values of ten independent experiments.

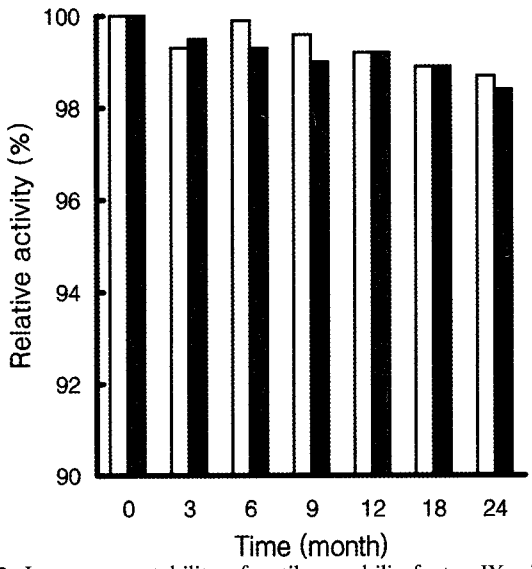

Fig. 2. Long-term stability of antihemophilic factor IX without (□) and with dry-heat treatment (■). These results are the mean values of three independent experiments.

Comparison of Physical and Biochemical Properties of Factor IX before and after Dry-Heat Treatment

In order to incorporate the dry-heat treatment into the manufacturing process, the physical and biochemical characteristics of factor IX should be validated as not changing during the process and that the dry-heat process does not induce any toxicity. The influence of dry-heat treatment at 100 ± 1°C for 30 min on the factor IX characteristics was studied (Table 2). The physical characteristics of the product, *i.e.* color and solubility of the powder, were not modified after dry-heat treatment. SDS-PAGE did not reveal the emergence of new bands (data not shown), indicating that the treatment had not induced cleavage. No abnormal toxicity against mice was

Table 3. Inactivation of HAV through lyophilization and dry-heat treatment

Sample	Total HAV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Before lyophilization	7.49 ± 0.12	–
After lyophilization	6.23 ± 0.18	1.26
10 min after dry heat	ND[a] (≤1.89[b])	≥5.60
20 min after dry heat	ND[a] (≤1.89[b])	≥5.60
30 min after dry heat	ND[a] (≤1.89[b])	≥5.60

These results are the mean values of three independent experiments.
[a]No infectious virus was detected.
[b]These values were calculated using a theoretical minimum detectable level of infectious virus, with a 95% confidence level.

Table 4. Inactivation of EMCV through lyophilization and dry-heat treatment

Sample	Total EMCV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Before lyophilization	7.97 ± 0.35	–
After lyophilization	3.16 ± 0.24	4.81
10 min after dry heat	ND[a] (≤1.89[b])	≥6.08
20 min after dry heat	ND[a] (≤1.89[b])	≥6.08
30 min after dry heat	ND[a] (≤1.89[b])	≥6.08

These results are the mean values of three independent experiments.
[a]No infectious virus was detected.
[b]These values were calculated using a theoretical minimum detectable level of infectious virus, with a 95% confidence level.

induced after dry-heat treatment. Also, the concentration of pyrogenic substances remained unchanged. These results indicate that the heated factor IX maintained the same physical and biochemical properties.

Long Term Stability of Factor IX after Dry-Heat Treatment

The influence on the long term stability of factor IX due to dry-heat treatment at 100 ± 1°C for 30 min was studied. Factor IX products, with or without dry-heat treatment, were stored at 4°C for 24 months, and their potencies periodically measured (Fig. 2). The factor IX activities of both products were stable up to 24 months.

Virus Inactivation during Lyophilization and Dry-Heat Treatment

It is known that virus infectivity substantially diminishes after being subjected to lyophilization as well as dry-heat treatment [19,27]. Therefore, the effects of both of these process on the inactivation of viruses were measured. HAV was potentially sensitive to the lyophilization process, and was inactivated from an initial titer of 7.49 log_{10} $TCID_{50}$ to 6.23 log_{10} $TCID_{50}$, with an average log reduction factor of 1.26. The remaining HAV after lyophilization was completely inactivated to undetectable levels within 10 min of dry-heat treatment (Table 3). EMCV was very sensitive to the lyophilization process,

Table 5. Inactivation of PPV through lyophilization and dry-heat treatment

Sample	Total PPV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Before lyophilization	6.65 ± 0.28	–
After lyophilization	5.75 ± 0.36	0.90
10 min after dry heat	4.80 ± 0.32	1.85
20 min after dry heat	4.54 ± 0.26	2.11
30 min after dry heat	4.01 ± 0.35	2.64

These results are the mean values of three independent experiments.

Table 6. Inactivation of BHV through lyophilization and dry-heat treatment

Sample	Total BHV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Before lyophilization	8.92 ± 0.40	–
After lyophilization	7.60 ± 0.50	1.32
10 min after dry heat	6.33 ± 0.21	2.59
20 min after dry heat	5.44 ± 0.12	3.48
30 min after dry heat	5.33 ± 0.14	3.59

These results are the mean values of three independent experiments.

and was inactivated from an initial titer of 7.97 log_{10} $TCID_{50}$ to 3.16 log_{10} $TCID_{50}$, with an average log reduction factor of 4.81. The remaining EMCV after lyophilization was completely inactivated to undetectable levels within 10 min of dry-heat treatment (Table 4). PPV was partially sensitive to the lyophilization process, and was inactivated from an initial titer of 6.65 log_{10} $TCID_{50}$ to 5.75 log_{10} $TCID_{50}$, with an average log reduction factor of 0.90. PPV was incompletely inactivated, with considerable remaining residual infectivity of 4.01 log_{10} $TCID_{50}$, even after 30 min of dry-heat treatment (Table 5). BHV was potentially sensitive to the lyophilization process, and was inactivated from an initial titer of 8.92 log_{10} $TCID_{50}$ to 7.60 log_{10} $TCID_{50}$, with an average log reduction factor of 1.32. During dry-heat treatment for 30 min, BHV was gradually inactivated from an initial titer of 7.60 log_{10} $TCID_{50}$ to 5.33 log_{10} $TCID_{50}$ (Table 6). The overall log reduction factors achieved through lyophilization and dry-heat treatment were ≥5.60 for HAV, ≥6.08 for EMCV, 2.64 for PPV, and 3.59 for BHV.

The result for EMCV, a model virus for HAV, showing its greater sensitive to lyophilization than that of HAV agrees well with the previous observations [19,28,29]. The log reduction factors for HAV and EMCV during lyophilization of antihemophilic factor VIII were 1.21 and 4.57, respectively. Also, the log reduction factors obtained during lyophilization of urokinase were 1.48 and 4.54 for HAV and EMCV, respectively. The mechanism by which lyophilization inactivates viruses is poorly understood, but might be due to the destabilization of the quaternary interactions between components of the capsid. Under normal conditions, water of solvation will be important in maintaining a protein's secondary structure, which may also be involved in hydrogen bond formation between different sub-units. This water would be re-

moved during the lyophilization process, which might result in disruption of normal protein interactions.

Parvovirus is known to be very resistant to many physicochemical agents [8,30]. Through this study, PPV, a model virus for the human parvovirus B19, was confirmed as being less susceptible than other viruses to lyophilization and dry-heat treatment, when comparing the log reduction factor of PPV with those of HAV, EMCV, and BHV.

The time required for the complete inactivation of HAV during dry-heat treatment was 10 min, and the log reduction factor obtained during the 10 min of treatment was ≥4.34. From this result, dry-heat treatment can be concluded to be a robust and effective step for the elimination of HAV. Since the viral load was reduced to undetectable levels within the first 10 min of the total 30 min of treatment, the extent of HAV inactivation was concluded to be several orders of magnitude greater than the potential HAV load of the current plasma pool.

CONCLUSION

To improve the viral safety of plasma-derived factor IX, with particular regards to HAV, a terminal dry-heat treatment process has been developed. The analysis of several operational batches showed that the process was consistent and reproducible. No batch-to-batch variation was observed in terms of the physico-chemical properties and recovery yields of factor IX. The results obtained lead us to conclude that dry-heat treatment at 100 ± 1°C for 30 min, combined with the solvent/detergent method, improves the viral safety of factor IX concentrate, without substantial loss of the biological activity or evident changes in the physical and biochemical characteristics.

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