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Dry-Heat Treatment Process for Enhancing Viral Safety of an Antihemophilic Factor VIII Concentrate Prepared from Human Plasma

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Viral safety is a prerequisite for manufacturing clinical antihemophilic factor VIII concentrates from human plasma. With particular regard 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 VIII concentrate. The loss of factor VIII activity during dry-heat treatment was of about 5%. No substantial changes were observed in the physical and biochemical characteristics of the dry-heat-treated factor VIII compared with those of the factor VIII before dry-heat treatment. The dry-heat-treated factor VIII 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, murine encephalomyocarditis virus (EMCV), and human immunodeficiency virus (HIV) were completely inactivated to below detectable levels within 10 min of the dry-heat treatment. Bovine herpes virus (BHV) and bovine viral diarrhea virus (BVDV) were potentially sensitive to the treatment. However porcine parvovirus (PPV) was slightly resistant to the treatment. The log reduction factors achieved during lyophilization and dry-heat treatment were ≥5.55 for HAV, ≥5.87 for EMCV, ≥5.15 for HIV, 6.13 for BHV, 4.46 for BVDV, and 1.90 for PPV. These results indicate that dry-heat treatment improves the virus safety of factor VIII 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 VIII, dry-heat treatment, hepatitis A virus, virus inactivation

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Hemophilia A is an inherited bleeding disorder, in which the blood clotting protein factor VIII is deficient or abnormal [10]. Until now, hemophilia has generally been treated by injection with coagulation factor concentrates, which are made from 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) [3, 14, 33]. The development of virus inactivation and/or removal technologies has greatly reduced the frequency of such transmissions [3, 22, 26]. 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 [35]. 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 [30]. Consequently, the blood industry is paying particular attention to systems capable of inactivating and/or removing non-enveloped viruses, such as hepatitis A virus (HAV) and human parvovirus B19 [5, 23, 25].

Recently, there have been a few reports describing HAV infections in hemophilic patients having received antihemophilic factor VIII concentrates prepared from large plasma pools using conventional chromatography procedures coupled with S/D treatment, although whether and how the implicated antihemophilic factor concentrates became contaminated with infectious HAV remain to be clarified [7, 12, 31]. Therefore, the international regulation for the validation of HAV safety has had to be re-inforced. 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 physicochemical inactivation [2].

The Green Cross Corp. is currently producing an antihemophilic factor VIII concentrate (GreenEight) using

conventional ion-exchange chromatography. The manufacturing process of GreenEight 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 regard to HAV, for improving the safety of the factor VIII.

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 order to ensure survival of the labile protein, which subsequently have to be removed from the products [18]. However, dry-heat treatment after lyophilization of the product can be performed with no stabilizers addition. Therefore, dry-heat treatment can be one of the choices generally recommended for virus inactivation [8, 28, 29, 32].

The aim of this study was to improve the safety of factor VIII concentrates, with regards to non-lipid-enveloped viruses. For this purpose, a systematic study for the development of a dry-heat process was conducted, 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 B19)), and three enveloped viruses (HIV, 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), and bovine viral diarrhea virus (BVDV; a model virus for human hepatitis C virus)).

Materials and Methods

Preparation of Factor VIII Solution

The factor VIII concentrate used in this study was a factor VIII complex prepared by conventional ion-exchange chromatography, with a solvent-detergent step for virus inactivation. The factor VIII complex was commercialized by Green Cross Corp., under the name GreenEight. The factor VIII 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 36 IU of factor VIII per milliliter. One IU of factor VIII is the amount contained in 1 ml of normal human plasma.

Biological, Physical, and Chemical Analyses

All the analyses were performed according to the Standard Operating Procedure (SOP), based on the Korean Pharmacopoeia, British Pharmacopoeia, and U.S. Pharmacopoeia. The factor VIII activity was determined using the clotting method with factor-VIII-deficient plasma. Clotting times were determined on a KC10 coagulometer (Amelung, Lemgo, Germany). The residual moisture content of the lyophilized factor VIII 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 VIII Activity

The effect of lyophilization on the activity of factor VIII was evaluated either with or without the addition of stabilizing agents. The stabilizing agents were 10 mM sodium citrate, 120 mM glycine, 1 mM calcium chloride, and 200 mM sodium chloride. Factor VIII solution was distributed in final containers at 10 ml/vial. The vials were loaded into a freeze dryer (Genesis 25XL; VirTis, NY, U.S.A.), which had been precooled to −40°C, and held for 6 h for pre-freezing. After switching the vacuum on, the temperature was maintained at −40°C for 4 h for stabilization. The temperature was then ramped to −20°C at 10°C/h, and held at this temperature for 30 h. The temperature was then ramped to 22°C at 5°C/h, and held at this temperature for 1 h. The temperature was then dropped to 19°C at 5°C/h, and held at this temperature for a further 11 h. The vials of freeze-dried factor VIII were closed under vacuum and capped before storage at 4°C. The factor VIII activities, both before and after lyophilization, were measured and the residual moisture content of lyophilized factor VIII was determined.

Effect of Dry-Heat Treatment on Factor VIII Activity

The effect of dry-heat treatment on the activity of factor VIII 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°C, 80±1°C, and 100±1°C, respectively. The temperature was continuously monitored in several reference bottles, and recorded using a Temperature Validator (Korea Science, Seoul, Korea). 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 VIII activity of the dry-heated products was 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 [11]. 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 VIII 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 VIII activity, and residual moisture content, the factor VIII solution, without the addition of virus, was filled into the vials. After lyophilization, the residual moisture content of the freeze-dried factor VIII was determined. Titers of viruses were measured before and after lyophilization. The lyophilized samples were loaded into a convection drying oven (Korea Science, Seoul, Korea), 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 six different model and relevant viruses; HAV (HM/175/18f

clone B, ATCC VR-1402), EMCV (ATCC VR-129B), PPV (ATCC VR-742), HIV (strain IIIB; Advanced Biotechnologies, Maryland, U.S.A.), BHV (ATCC VR-188), and BVDV (ATCC VR-534). For the propagation and titration of HAV, EMCV, PPV, HIV, BHV, and BVDV, FRhK-4 (ATCC CRL-1688), Vero cells (ATCC CCL-81), Minipig kidney (MPK) cells (ATCC CCL-166), C8166 cells (European Collection of Animal Cell Culture), Madin-Derby bovine kidney (MDBK) cells (ATCC CRL-22), and bovine turbinate (BT) cells (ATCC CRL-1390) were respectively used, as described in previous reports [16, 17, 20, 34]. All the cells except C8166 cells were grown in a high-glucose Dulbecco's modified Eagle's medium (HG DMEM) containing 2% fetal bovine serum (FBS). C8166 cells were grown in RPMI1640 medium containing 2% FBS.

An aliquot of 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 [13]. For the titration of HAV, EMCV, PPV, BHV, and BVDV, 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 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. For the 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 h, and the wells were fed with 0.1 ml of the tissue culture medium. Approximately 14–21 days later, the wells were examined for any cytopathic effect and syncytial formation. 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 that 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. 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 a previous report [19]. All the virus inactivation experiments were carried out three times. Mean values and standard deviations of three independent experiments were evaluated using the Microsoft Excel program.

RESULTS AND DISCUSSION

Factor VIII Activity and Residual Moisture After Lyophilization

The instability of proteins is found to be one of the major constraints in the development of biopharmaceuticals [9].

Table 1. Factor VIII activity before and after lyophilization.

Sample	Factor VIII (IU/ml)
Before lyophilization	35.5±1.7
After lyophilization	
Without stabilizing agents	16.4±3.2
With stabilizing agents	33.9±0.5

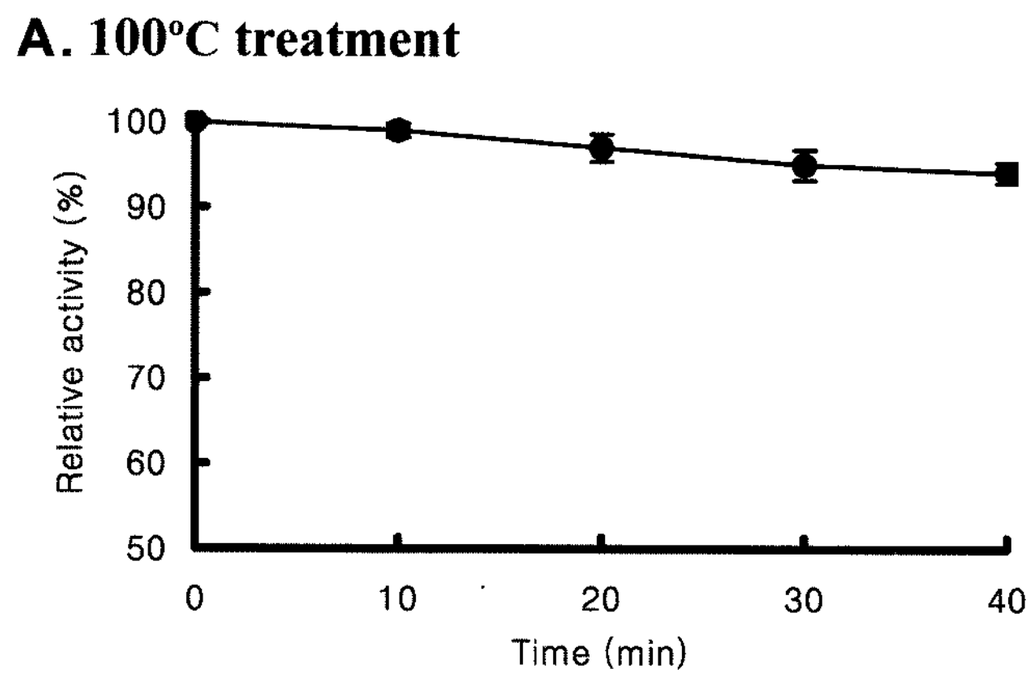
These results are the mean values of 10 independent experiments.

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 [6]. Without appropriate stabilizing excipients, most protein preparations are at least partially denatured owing 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 VIII during lyophilization, 10 mM sodium citrate, 120 mM glycine, 1 mM calcium chloride, and 200 mM sodium chloride were chosen as stabilizing agents. The factor VIII activities, both before and after lyophilization, were measured (Table 1). The loss of factor VIII activity in the absence of stabilizing agents was more than 54% after lyophilization. However, a slight drop in the factor VIII activity, within the range of 5%, was observed in the presence of the excipients. This small loss of factor VIII activity during lyophilization was clearly due to the addition of the stabilizing excipients to the factor VIII 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 [27]. The Korean Pharmacopoeia has recommended that the moisture content of lyophilized biopharmaceuticals, including antihemophilic factor VIII, should be below 3%. Therefore, the moisture contents of the lyophilized products were measured. The mean (N=5) residual moisture value in lyophilized factor VIII was 0.69%.

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

Although there have been a few reports on the evaluation of the virucidal effect of dry-heat treatment [8, 28, 29, 32], less has been reported about the effect of dry-heat conditions on the physicochemical and biological properties of factor VIII. Dry-heat treatments at 100°C for 30 min, 80°C for



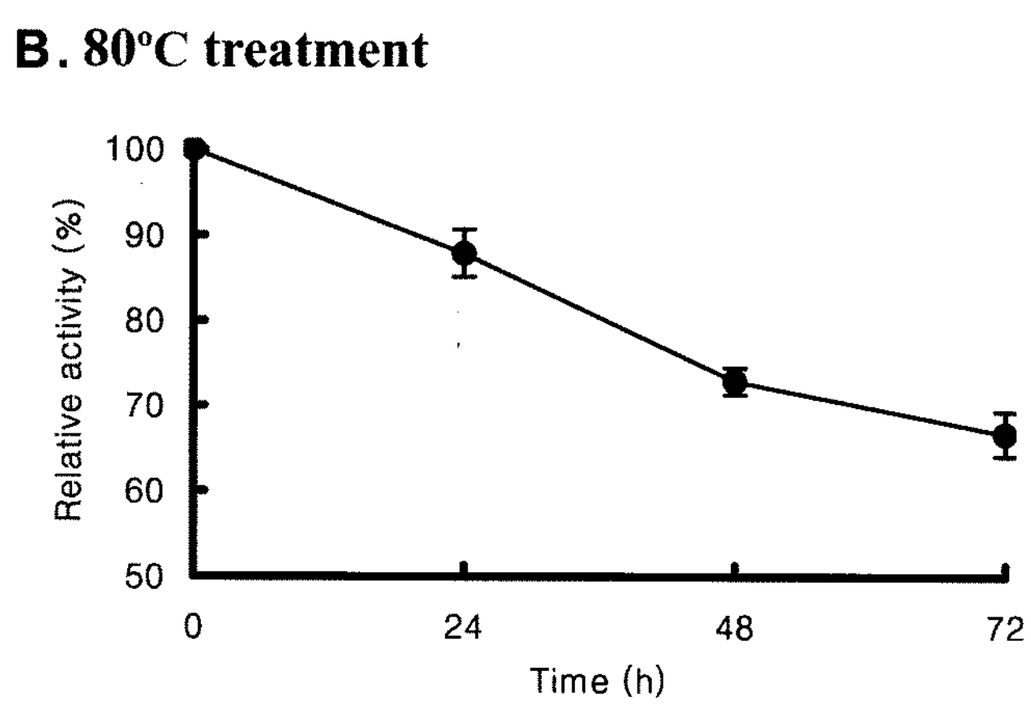


Fig. 1. Effect of dry-heat temperature on the activity of antihemophilic factor VIII.
These results are the mean values of three independent experiments.

72 h, or 60°C for 144 h have been generally recommended for improving the virus safety of biopharmaceuticals. Therefore, the effect of the dry-heat temperature, at 100, 80, and 60°C, was examined on the activity of the lyophilized factor VIII as a function of time (Fig. 1). The activity of the factor VIII decreased during dry-heat treatment. The loss of factor VIII activity during the treatment at 100±1°C for 30 min was less than 5%. However, the losses during the treatments at 80±1°C for 72 h and 60±1°C for 144 h were about 32% and 28%, 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 VIII.

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

Items	Before treatment	After treatment
Activity (IU/vial)	277.1±8.4	273.2±14.7
Moisture content (%)	0.69±0.24	0.68±0.08
Clottable protein (mg/IU)	0.004±0.0003	0.002±0.0002
pH	7.03±0.06	6.99±0.10
Solubility (min)	1.8±0.3	1.9±0.2
Nonsoluble materials	Negative	Negative
Pyrogens (°C)	0.44±0.25	0.64±0.08
Abnormal toxicity against mice	Negative	Negative

These results are the mean values of five independent experiments.

Comparison of Physical and Biochemical Properties of Factor VIII 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 VIII 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 VIII 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 induced after dry-heat treatment. In addition, the concentration of pyrogenic substances remained unchanged. Moreover, no batch-to-batch variation was observed in terms of the physicochemical properties and recovery yields of factor VIII. These results indicate that the heated factor VIII maintained the same physical and biochemical properties.

In the previous report, dry-heat treatment at 100±1°C for 30 min did not induce substantial loss of the biological activity or evident changes in the physical and biochemical characteristics of factor IX [32]. Therefore, it was concluded that dry-heat treatment can be an alternative of pasteurization to improve the viral safety of the labile protein therapeutics.

Long-Term Stability of Factor VIII After Dry-Heat Treatment

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

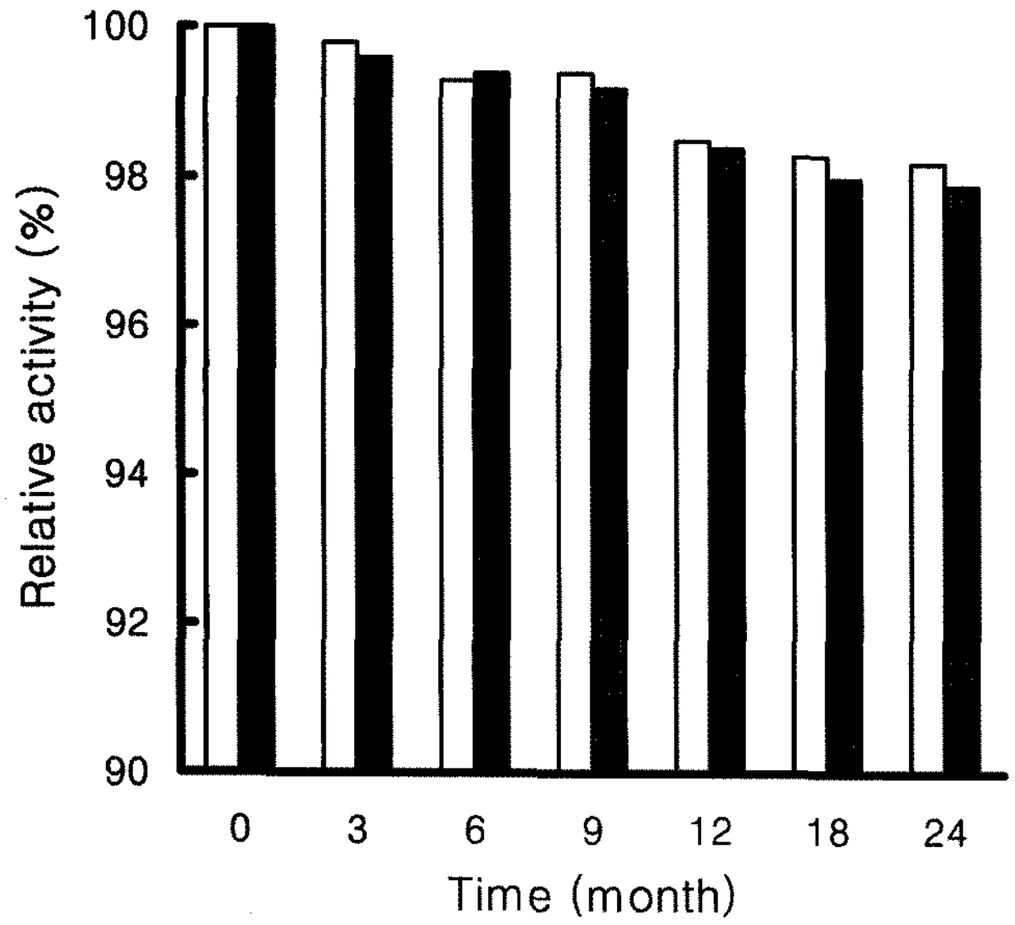


Fig. 2. Long-term stability of antihemophilic factor VIII without (□) and with (■) dry-heat treatment.
These results are the mean values of three independent experiments.

without dry-heat treatment, were stable up to 24 months at 4°C [32].

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, 21]. Therefore, the effects of both of these processes on the inactivation of viruses were measured. HAV was potentially sensitive to the lyophilization process, and was inactivated from an initial titer of 7.44 $\log_{10}$ $TCID_{50}$ to 6.23 $\log_{10}$ $TCID_{50}$, with an average log reduction factor of 1.21. 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, and was inactivated from an initial titer of 7.76 $\log_{10}$ $TCID_{50}$ to 3.69 $\log_{10}$ $TCID_{50}$, with an average log reduction factor of 4.07. The remaining EMCV after lyophilization was completely

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

Sample	Total HAV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Before lyophilization	7.44±0.36	
After lyophilization	6.23±0.34	1.21
10 min after dry heat	ND[a] (≤1.89[b])	≥5.55
20 min after dry heat	ND[a] (≤1.89[b])	≥5.55
30 min after dry heat	ND[a] (≤1.89[b])	≥5.55

[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	7.76±0.43	–
After lyophilization	3.69±0.51	4.07
10 min after dry heat	ND[a] (≤1.89[b])	≥5.87
20 min after dry heat	ND[a] (≤1.89[b])	≥5.87
30 min after dry heat	ND[a] (≤1.89[b])	≥5.87

[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.

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 5.91 $\log_{10}$ $TCID_{50}$ to 5.22 $\log_{10}$ $TCID_{50}$, with an average log reduction factor of 0.69. 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). HIV was potentially sensitive to the lyophilization process, and was inactivated from an initial titer of 6.50 $\log_{10}$ $TCID_{50}$ to 5.32 $\log_{10}$ $TCID_{50}$, with an average log reduction factor of 1.18. The remaining HIV after lyophilization was completely inactivated to undetectable levels within 10 min of dry-heat treatment (Table 6). BHV was potentially sensitive to the lyophilization process, and was inactivated from an initial titer of 8.66 $\log_{10}$ $TCID_{50}$ to 7.39 $\log_{10}$ $TCID_{50}$, with an average log reduction factor of 1.27. During dry-heat treatment for 30 min, BHV was gradually inactivated from an initial titer of 7.39 $\log_{10}$ $TCID_{50}$ to 2.53 $\log_{10}$ $TCID_{50}$ (Table 7). BVDV was potentially sensitive to the lyophilization process, and was inactivated from an initial titer of 5.70 $\log_{10}$ $TCID_{50}$ to 3.96 $\log_{10}$ $TCID_{50}$, with an average log reduction factor of 1.74. During dry-heat treatment for 30 min, BVDV was gradually inactivated from an initial titer of 3.96 $\log_{10}$ $TCID_{50}$ to 1.24 $\log_{10}$ $TCID_{50}$ (Table 8). The overall log reduction factors achieved through lyophilization and dry-heat treatment were ≥5.55 for HAV, ≥5.87 for EMCV, 1.90 for PPV, ≥5.15 for HIV, 6.13 for BHV, and 4.46 for BVDV.

The result for EMCV, a model virus for HAV, showing its greater sensitive to lyophilization than that of HAV

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

Sample	Total PPV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Before lyophilization	5.91±0.47	–
After lyophilization	5.22±0.28	0.69
10 min after dry heat	4.80±0.24	1.11
20 min after dry heat	4.27±0.26	1.64
30 min after dry heat	4.01±0.42	1.90

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

Sample	Total HTV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Before lyophilization	6.50±0.22	–
After lyophilization	5.32±0.18	1.18
10 min after dry heat	ND[a] (≤1.35[b])	5.15
20 min after dry heat	ND[a] (≤1.35[b])	5.15
30 min after dry heat	ND[a] (≤1.35[b])	5.15

[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.

agrees well with the previous observations [15, 19, 32]. The log reduction factors for HAV and EMCV during lyophilization of antihemophilic factor IX were 1.26 and 4.81, respectively. In addition, 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 subunits. This water would be removed during the lyophilization process, which might result in disruption of normal protein interactions.

Parvovirus is known to be very resistant to many physicochemical agents [4, 23]. 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 other viruses. This result agrees well with the previous report [32]. The log reduction factor for PPV achieved during lyophilization and dry-heat treatment of factor IX was 2.64; however, those were ≥5.60 for HAV, ≥6.08 for EMCV, and 3.59 for BHV.

The risk of plasma pool contamination with infectious HAV is difficult to calculate because an acute HAV infection shows a limited, short, and low viremic period of about 2 weeks with a maximum 10^4 to 10^6 infectious particles per milliliter of blood. Post-transfusion hepatitis A is also very uncommon [24]. Considering the fact that all the plasma for the manufacture of factor VIII 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, the possible titer of HAV contaminated in the plasma pool should be very low. 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 inferred to be several orders of magnitude greater than the potential HAV load of the current plasma pool.

Taking into account that several production steps such as cryoprecipitation and chromatography procedures contribute to the removal of HAV, it can be assumed that terminal dry-heat-treated factor VIII is safe against HAV. The analysis of several operational batches showed that the dry-heat treatment process was consistent and reproducible. No batch-to-batch variation was observed in terms of the physicochemical properties and recovery yields of factor VIII. The results obtained through this study 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 VIII concentrates, without substantial loss of the biological activity or evident changes in the physical and biochemical characteristics.

Table 7. Inactivation of BHV through lyophilization and dry-heat treatment.

Sample	Total BHV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Before lyophilization	8.66±0.45	–
After lyophilization	7.39±0.46	1.27
10 min after dry heat	5.63±0.43	3.03
20 min after dry heat	3.90±0.32	4.76
30 min after dry heat	2.53±0.38	6.13

Table 8. Inactivation of BVDV through lyophilization and dry-heat treatment.

Sample	Total BVDV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Before lyophilization	5.70±0.38	–
After lyophilization	3.96±0.32	1.74
10 min after dry heat	3.53±0.22	2.17
20 min after dry heat	2.90±0.24	2.80
30 min after dry heat	1.24±0.12	4.46

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References

1. Addiego, Jr. J. E., E. Gomperts, S. Liu, P. Bailey, S. G. Courter, M. L. Lee, G. G. Neslund, H. S. Kingdon, and M. J. Griffith. 1992. Treatment of hemophilia A with a highly purified factor VIII concentrate prepared by anti-FVIIIc immunoaffinity chromatography. *Thromb. Haemost.* **67:** 19–27.

2. Andrewes, C. 1989. *Andrewes' Viruses of Vertebrates*, pp. 120–145. Balliere Tindal, London, U.K.
3. Berkman, S. A. 1988. Infectious complications of blood transfusion. *Blood Rev.* **2:** 206–210.
4. Boschetti, N., I. Niederhauser, C. Kempf, A. Stuhler, J. Lower, and J. Blumel. 2004. Different susceptibility of B19 virus and mice minute virus to low pH treatment. *Transfusion* **44:** 1079–1086.
5. Burnouf, T. and M. Radosevich. 2000. Reducing the risk of infection from plasma products: Specific preventative strategies. *Blood Rev.* **14:** 94–110.
6. Carpenter, J. F., M. J. Pikal, B. S. Chang, and T. W. Randolph. 1997. Rational design of stable lyophilized protein formulations: Some practical advice. *Pharm. Res.* **14:** 969–975.
7. Chudy, M., I. Budek, B. Keller-Stanislawski, K. A. McCaustland, S. Neidhold, B. H. Robertson, C. M. Nubling, R. Seitz, and J. Lower. 1999. A new cluster of hepatitis A infection in hemophiliacs traced to a contaminated plasma pool. *J. Med. Virol.* **57:** 91–99.
8. Dichtelmuller, H., D. Rudnick, B. Breuer, R. Kotitschke, M. Kloft, A. Darling, *et al.* 1996. Improvement of virus safety of a S/D treated factor VIII concentrate by additional dry heat treatment at 100°C. *Biologicals* **24:** 125–130.
9. Gummadi, S. N. 2003. What is the role of thermodynamics on protein stability? *Biotechnol. Bioprocess Eng.* **8:** 9–18.
10. Hoyer, L. W. 1994. Hemophilia A. *N. Engl. J. Med.* **330:** 38.
11. International Conference on Harmonisation. 1998. Guidance on viral safety evaluation of biotechnology products derived from cell lines of human or animal origin; availability. *Federal Register* **63:** 51074–51084.
12. Jee, Y. M., U. Go, D. Cheon, Y. Kang, J. D. Yoon, S. W. Lee, *et al.* 2006. Detection of hepatitis A virus from clotting factors implicated as a source of HAV infection among haemophilia patients in Korea. *Epidemiol. Infect.* **134:** 87–93.
13. Kärber, J. 1931. Beitrag zur kollectiven Behandlung pharmakologische Reihenversuche. *Arch. Exp. Path. Pharmak.* **162:** 480–483.
14. Kim, H., Y. Jee, H.-S. Mun, B.-C. Song, J.-H. Park, J.-W. Hyun, *et al.* 2007. Comparison of full genome sequences between two hepatitis B virus strains with or without preC mutation (A1896) from a single Korean hepatocellular carcinoma patient. *J. Microbiol. Biotechnol.* **17:** 701–704.
15. Kim, I. S. 2003. Comparative inactivation of hepatitis A virus and murine encephalomyocarditis virus to various inactivation processes. *Kor. J. Microbiol.* **39:** 242–247.
16. Kim, I. S., H. G. Eo, C. W. Park, C. E. Chang, and S. Lee. 2001. Removal and inactivation of human immunodeficiency virus (HIV-1) by cold ethanol fractionation and pasteurization during the manufacture of albumin and immunoglobulins from human plasma. *Biotechnol. Bioprocess Eng.* **6:** 25–30.
17. Kim, I. S., Y. W. Choi, and S. R. Lee. 2004. Optimization and validation of a virus filtration process for efficient removal of viruses from urokinase solution prepared from human urine. *J. Microbiol. Biotechnol.* **14:** 140–147.
18. Kim, I. S., Y. W. Choi, S. R. Lee, and H. M. Sung. 2004. Cold ethanol fractionation and heat inactivation of hepatitis A virus during manufacture of albumin from human plasma. *Biotechnol. Bioprocess Eng.* **9:** 57–60.
19. Kim, I. S., Y. W. Choi, S. R. Lee, H. S. Woo, and S. Lee. 2001. Removal and inactivation of viruses during manufacture of a high purity antihemophilic factor VIII concentrate from human plasma. *J. Microbiol. Biotechnol.* **11:** 497–503.
20. Kim, I. S., Y. W. Choi, S. R. Lee, M. S. Lee, K. H. Huh, and S. Lee. 2001. Removal and inactivation of hepatitis A virus during manufacture of a high purity antihemophilic factor VIII concentrate from human plasma. *J. Microbiol.* **39:** 67–73.
21. Kim, I. S., Y. W. Choi, S. R. Lee, Y. Kang, K. M. Lee, D. H. Park, H. S. Woo, and S. Lee. 2002. Removal and inactivation of hepatitis A virus during manufacture of urokinase from human urine. *Biotechnol. Bioprocess Eng.* **7:** 340–346.
22. Klein, H. G. 2005. Pathogen inactivation technology: Cleansing the blood supply. *J. Intern. Med.* **257:** 224–237.
23. Laub, R. and P. Strengers. 2002. Parvovirus and blood products. *Pathol. Biol. (Paris)* **50:** 339–348.
24. Lemon, S. M. 1994. The natural history of hepatitis A: The potential for transmission by transfusion of blood or blood products. *Vox Sang.* **67(Suppl 4):** 19–23.
25. Morgenthaler, J. J. 2000. New development in plasma fractionation and virus inactivation. *Vox Sang.* **78(Suppl 2):** 217–221.
26. Mosley, J. W. and J. Rakela. 1999. Foundling viruses and transfusion medicine. *Transfusion* **39:** 1041–1044.
27. Murgatroyd, K. 1997. The freeze drying process, pp. 1–58. *In* P. Cameron (ed.), *Good Pharmaceutical Freeze-Drying Practice.* Interpharm Press, IL, U.S.A.
28. Powell J. S., M. Bush, J. Harrison, C. Abildgaard, E. Vosburgh, A. R. Thompson, and D. Hurst. 2000. Safety and efficacy of solvent/detergent-treated antihaemophilic factor with an added 80°C terminal dry heat treatment in patients with haemophilia A. *Haemophilia* **6:** 140–149.
29. Roberts, P. L. and H. Hart. 2000. Comparison of the inactivation of canine and bovine parvovirus by freeze-drying and dry-heat treatment in two high purity factor VIII concentrates. *Biologicals* **28:** 185–188.
30. Robertson, B. H. and D. D. Erdman. 2000. Non-enveloped viruses transmitted by blood and blood products. *Dev. Biol. Stand.* **102:** 29–35.
31. Robinson, S. M., H. Schwinn, and A. Smith. 1992. Clotting factors and hepatitis A. *Lancet* **340:** 1465.
32. Shin, J. S., Y. W. Choi, H. M. Sung, Y.-W. Ryu, and I. S. Kim. 2006. Enhanced virus safety of a solvent/detergent-treated antihemophilic factor IX concentrate by dry-heat treatment. *Biotechnol. Bioprocess Eng.* **11:** 19–25.
33. Shin, K.-S., J. H. Lim, J.-H. Kim, H. Myung, and S.-W. Lee. 2006. Inhibition of the replication of hepatitis C virus replicon with nuclease-resistant RNA aptamers. *J. Microbiol. Biotechnol.* **16:** 1634–1639.
34. Song, M.-S., Y. Joo, E. H. Lee, J.-Y. Shin, C.-J. Kim, K.-S. Shin, M. H. Sung, and Y. K. Choi. 2006. Genetic characterization of encephalomyocarditis virus isolated from aborted swine fetus in Korea. *J. Microbiol. Biotechnol.* **16:** 1570–1576.
35. Tabor, E. 1999. The epidemiology of virus transmission by plasma derivatives: Clinical studies verifying the lack of transmission of hepatitis B and C viruses and HIV type 1. *Transfusion* **39:** 1160–1168.