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THE ROLE OF VARIOUS STABILIZERS IN THE ACQUISITION OF THERMO-TOLERANCE IN FACTOR VIII ACTIVITY

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ABSTRACT

We prepared a highly purified and relatively heat stable form of factor VIII which contained 25 units per mL (u/mL) activity using PEG-4000 and developed an effective and new manufacturing process. Heat treatment was performed at 80°C for 72 hrs in the presence of different stabilizers. In our studies, we used different organic solvents as preservatives to maintain factor VIII activity, since few enzymes have been observed to function perfectly well in such conditions. Our results show that the activity of factor VIII in the presence of organic solvent at 80°C for 72 hrs as viral inactivation is significantly decreased. It seems that irreversible aggregation of protein is the major mechanism in cessation of factor VIII activity. On the other hand, protein stability may be increased by certain low molecular weight substances. We therefore used different sugars and amino-acids to protect factor VIII activity in the process of heat treatment. Our results showed that glycine, glucose, saccharose, mannitol and BSA were not effective stabilizers of factor VIII. But the loss of factor VIII activity at 80°C for 72 hrs, in the presence of 10, 15 and 20% concentrations of trehalose, was about 15-20%, which is acceptable for large-scale processes. Thus, the condition obtained in the present study seems to be a more suitable condition than that reported previously.

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INTRODUCTION

Infection of a large number of hemophilic patients with hepatitis and HIV, transmitted by large-pooled clotting factors, has prompted the development of virucidal methods for inactivating infectious agents that escape plasma screening. Most virucidal methods are based on various types of heating procedures, i.e., lyophilized state in aqueous solution with stabilizers, and inorganic solvent or based on the use of solvent-detergent (S/D) methodologies.

Unheated factor VIII (FVIII) has been the main source of transmitting non-A, non-B hepatitis to almost all patients receiving treatment.[1-4]

It has also become evident that some viruses still escape the presently applied inactivation methods due to their resistance against both organic solvents and heat at 60°C. In this context human parvovirus B19 and hepatitis A virus are of particular concern.[5]

Heat treatment of FVIII at 80°C has proved to be the most effective and widely used method for inactivation of enveloped and non-enveloped viruses in the United Kingdom.[6] Follow-up studies performed for several years on 32 patients treated with these concentrates demonstrated that there was no apparent rise in liver enzymes in these patients, and neither HIV nor HBS seroconversions were reported.[7] However, it is well known that S/D treatment can

not be applied to non-enveloped viruses. In order to maintain the biological function of the more labile proteins, some additional stabilizers like amino-acids, citrate, sugars, etc. must be used. FVIII is one of the proteins which easily loses its activity.

Freeze-dried labile proteins tolerate temperatures up to 68°C reasonably well. The composition of the product, and mainly its moisture content, the type and quality of stabilizer and the method of freeze-drying, influence both the stability of protein and the degree of virus inactivation.

This paper studies the FVIII concentrate derived from pooled cryoprecipitate using a heat treatment viral inactivation technique followed by a different stabilizer.

MATERIALS AND METHODS

Purification of FVIII

Purified and heat treated FVIII is produced according to Fig. 1. As the purification procedure has already been described elsewhere,[8] a short description will be given as follows.

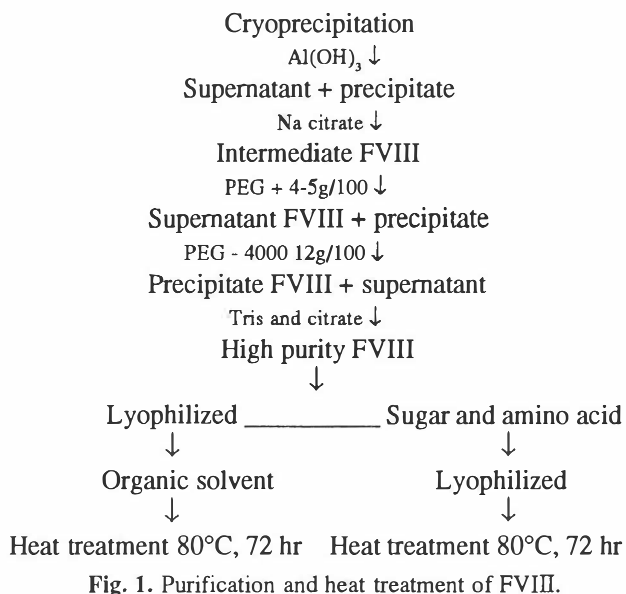

Fig. 1. Purification and heat treatment of FVIII.

The intermediate FVIII was obtained after absorption of FVIII-rich supernatant with $Al(OH)_3$. Then centrifugation at 20,000 rpm was performed. The purified FVIII obtained after intermediate FVIII was mixed with PEG, which is sufficient to precipitate most of the contaminant proteins and fibrinogen.

Heat treatment process

The purified FVIII obtained after PEG was mixed with different amounts of amino-acids and sugars (trehalose, glycine, mannitol, bovine serum albumin, 1% up to 50%) as stabilizers to protect FVIII activity against inactivation during the subsequent heat treatment.

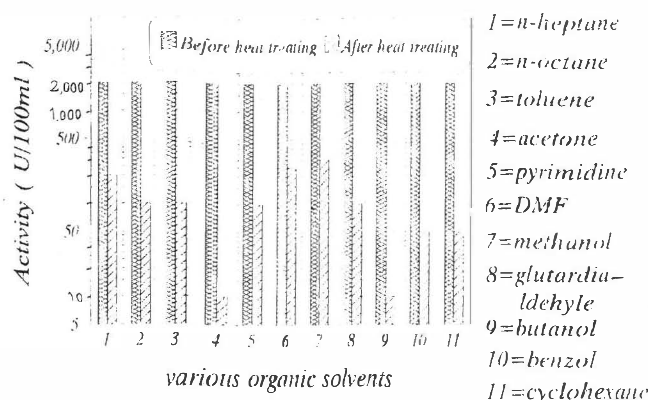

Fig. 2. Heat treatment of FVIII at 80°C for 72 hrs in the presence of organic solvents.

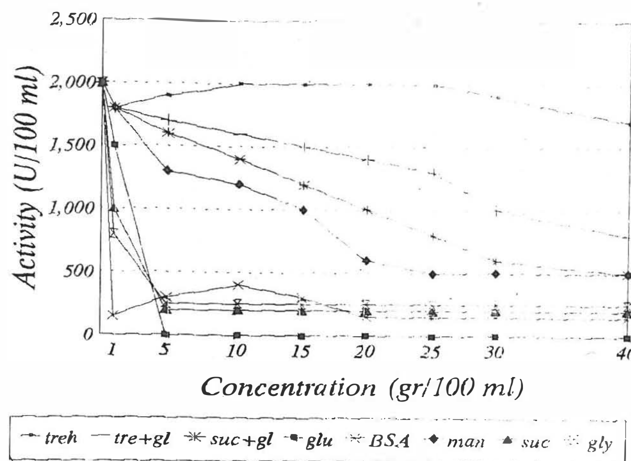

Fig. 3. Heat treatment of FVIII in the presence of different stabilizers.

The mixture was frozen and lyophilized, then incubated at 80°C for 72 hrs for the heat treatment.

The purified FVIII obtained after PEG was lyophilized, then mixed with different organic solvents such as glutardialdehyde, DMF, butanol, cyclohexane, toluene, methanol, chloroform, acetone, benzol, n-octane, n-heptane, and pyrimidine to protect FVIII activity against inactivation during the subsequent treatment at 80°C for 72 hrs.

Virus assays

Virus studies were performed in cooperation with the Virology Center of Rasool Akram Hospital and the Virology Laboratory of the Blood Transfusion Service. The rate and extent of inactivation of polio, measles and herpes viruses, added to the purified FVIII prior to heat treatment, were determined and compared to those obtained after heat treatment. Viruses were added at a 1:20 dilution to F VIII samples, and the samples were lyophilized and heat treated. For titration purposes vials were reconstituted in 1 mL of sterilized, distilled water and further, ten-fold serial dilutions were made in MEM medium before inoculation on specific

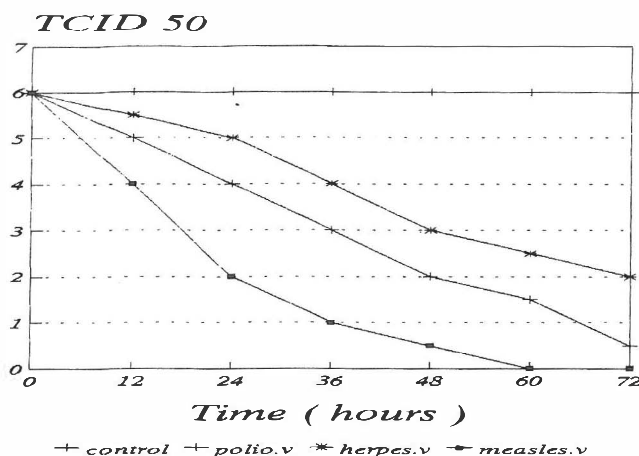

Fig. 4. Inactivation of viruses in FVIII by heat treatment at 80°C for 72 hrs.

cells. The infectivity of viruses were assessed by end-point dilution. Ten-fold serial dilutions were made in the culture medium. 0.1 mL of each dilution was used to inoculate 2 wells of cells in a 96-well microtiter plate and were incubated at 37°C. Cytopathic effects of viruses were scored after 48, 72, and 96 hrs and the TCID 50 value was calculated using the Spearman-Karber method.[9]

FVIII activity assay

Clotting assay was performed by one-stage activated thromboplastin time, using FVIII deficient human plasma as substrate and the cryoglobulin standard (Immuno, Austria).[10]

Biological parameters

Total protein was determined by the Biuret method, and potassium and sodium were determined by flame photometry. The fibrinogen content (clottable protein) was assayed by the method of Johnson et al.[11]

RESULTS

In vitro evaluation of FVIII concentrate

The biochemical characteristics of highly purified FVIII concentrate, produced by the above-mentioned procedure, are summarized in Table I.

Influence of organic solvent on thermal stability of FVIII

As shown in Fig. 2, heat treatment at 80°C for 72 hours in the presence of organic solvents was not successful. The loss of FVIII activity was from 80% to 100%. The physiological characteristics of the product, i.e., the color and solubility of the powder, were modified after heat treatment.

Table I. Characteristics of highly purified FVIII.

Characteristic	Contents
Protein (g/100)	1.12
FVIII: c (Iu/mL)	20-30
Fibrinogen (g/100)	<2
Osmolarity (mol/L)	<2
pH	7 + 0.2
Na (mmol/L)	50
K (mmol/L)	2.8
Cl	39
Yield	50%

Influence of amino acids and sugars on thermal stability of FVIII

To determine the effect of amino acids and sugars on the thermo-tolerance of FVIII, various concentrations of these substances were added to the product before lyophilization, the results are shown in Fig. 3. The loss of FVIII activity in the presence of glucose (glu), sucrose (suc), and glycine (gly) was about 90%. So they are not suitable to be used for thermostability of FVIII; but using mannitol (man) and BSA, the loss of activity was 40-50%. Previously, the authors have shown that physiological concentrations of trehalose efficiently stabilize proteins against thermal denaturation *in vitro*, and that disaccharides give better results than other micromolecules. As shown in Fig. 3, trehalose concentrations between 10 to 20% enhanced the thermal stability of FVIII in viral inactivation, so it is a very good stabilizer for safe viral inactivation of FVIII at 80°C for 72 hours.

Virucidal efficacy

The inactivation kinetics of the polio virus as a model for HAV (small non-enveloped viruses), the measles virus as a model for HIV and HCV (medium/large enveloped RNA viruses), and herpes virus 1 as a model for HBV (large enveloped DNA viruses), by heat treatment in lyophilized FVIII are shown in Fig. 4. After heat treatment at 80°C the virus titer was found to be reduced about 5 log and the viruses were no longer detectable. The non-heated virus control held at room temperature showed no significant virus inactivation.

DISCUSSION

In our studies we prepared a highly purified form of FVIII with a preparation activity of 25 units FVIII per mL

(25 u/mL) using PEG-4000. We successfully separated FVIII from fibrinogen and other contaminant proteins. During the last decade, an impressive amount of work on the study of virus inactivation has been fruitfully carried out. Several methods have been developed and their efficacy has been proven in experimental studies, clinical trials and even long term clinical use.[13]

Several manufacturers have used a protocol of heating at 68°C for 12 hrs, or 60°C for 72 hrs in dry state or 60°C for 2 hrs in humid media. These treatments are safe for HIV (because it is heat sensitive) but are not safe for NANB or HBV, and clinical investigation indicates that these methods are not completely safe.[13] We developed an effective new manufacturing process using 80°C heat for 72 hrs. The clinical safety of the product was obtained according to the standard.[14]

However, careful formulation such as moisture content, type and quality of stabilizers, and using a good method of freeze drying are major factors for successful heat treatment at 80°C. For protection of FVIII activity at 80°C and for 72 hrs, we evaluated various types of stabilizers.

1-Organic solvent is a new method for increasing the stability of FVIII in solution. This method is based on the fact that some enzymes and proteins have good activity in organic media.

A common feature of the inactivating covalent reactions is the participation of water. Some enzymes and proteins can show an increase in thermal stability in organic media. This enhanced stability occurs without deliberate change of the protein molecules.[15] However, in this study we used various organic solvents to stabilize FVIII against heat treatment at 80°C for 72 hrs. Our results were not successful for protection of FVIII against heat treatment at 80°C for 72 hrs. Studies on this enzyme (FVIII) indicate that aggregation was the major cause of inactivation. This is also the case for other enzymes, such as hydrolase, RNase, chymotrypsin and lysozyme.[15]

2- Protein stability may be increased by inclusion of certain low molecular weight substances. These additives can be different types and include solvent, polyols and sugars, and inert polymers sure as polyethylene glycol. Solutes are divided into osmolytics and ionic stabilizers.

Osmolytics are uncharged and affect solvent viscosity and surface tension and include polyols, polysaccharides and amino acids. Ionic stabilizers seem to act by shielding the surface charge. Additives can greatly enhance stability without chemical or genetic manipulation of the target protein.[15] However, we used different sugars and amino acids to protect FVIII activity in heat treatment. Our results showed that glycine, glucose, and saccharose were not effective for FVIII stability, and the loss of activity was about 80-90%, but mannitol and BSA were effective with only 30-40% loss. Nevertheless, these additives could not be useful in large-scale processes. In our experiment, the loss of FVIII activity at 80°C for 72 hrs in the presence of 10, 15, and 20% concentrations of trehalose was about 15-20%, which is acceptable for large-scale processes. Many authors suggested that trehalose is a stress-related metabolite of the cell that may contribute to induced thermo-tolerance.[16]

This hypothesis was initially based on the finding that trehalose level and thermo-tolerance are correlated under a wide variety of experimental conditions, and it has been shown by genetic methods that trehalose contributes to the thermo-tolerance of both *E. coli* and other microorganisms. The influence of trehalose is supposed to be a major determinant of thermo-tolerance, namely protein stability at high concentrations.

Physiological concentrations of trehalose were effective in protecting a number of widely different proteins against *in vitro* thermal inactivation.

Recently, some authors have tried to use trehalose as a stabilizer in coagulation factors, but the results have not yet been published.[12] In this study, we can conclude that trehalose is an effective agent for stabilizing FVIII against heating at 80°C for 72 hrs and in concentrations between 10 to 20%, enhances the thermal stability of FVIII in viral inactivation. Trehalose also reduced the heat-induced formation of protein aggregates. So it was at least a better protein stabilizer than any of a number of other compatible solutes, and is therefore a useful additive in industrial processes.

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REFERENCES

1. Mannucci PM, Colombo M: Virucidal treatment of clotting factor concentrates. Lancet 2: 782-5, 1988.
2. Brettler DB, Levine PH: Factor concentrates for treatment of hemophilia; which one to choose. Blood 73: 2067-73, 1989.
3. Schimp K, Mannucci PM, Kreuty W: Absence of hepatitis after treatment with a pasteurized FVIII. N Engl J Med 316: 918-922, 1987.
4. Mannucci PM: Schimp K, Abe T: Low risk of viral infection after administration of vapor-heated FVIII concentrate. Transfusion 32: 134-38, 1992.
5. Schwinn H, Stadler M: Josic DJ, Bal F: A solvent/detergent treated, pasteurised and highly purified FVIII concentrate: Arzne, For/Day Res 44(1): 188-91, 1994.
6. Winkelman L, Feldman PA, Evans DR: Severe heat treatment of lyophilized coagulation factor concentrates Curr Stud Hematol Transf 56: 55-65, 1989.

7. Colvin BT, Rizza CR, Hill FGH: Effect of dry heating oi coagulation factor concentrates at 80°C for 72 hrs on transmission of non-A non-B hepatitis. Lancet Oct. 8: 814-816, 1988.
8. Zandieh T: Purification and virus inactivation of FVIII. Ph.D. Thesis, Pasteur Institute of Iran, 2, 49-51, 1998.
9. Horowitz B, Wiele ME, Lippin A, Stryker MH: Inactivation of viruses in labile blood derivatives. 1: Disruption of lipid-enveloped viruses by tri(n) butyl phosphate detergent combination. Transfusion 25: 516-522, 1985.
10. Bowie EJW, Thompson JH, Didishein P, Owen CA: Mayo Clinical Laboratory Manual of Hemostasis. Philadelphia: W.B. Saunders, pp. 3, 87-95, 1971.
11. Johnson AJ, Tse AO, Newman J: Fibrinolytic and biochemical methods for the study of thrombolysis in man. In: Tocatin LM, Razal LA, (eds.), Application and Standardization: Blood, Coagulation, Hemorrhage and Thrombosis. New York: Grune and Stratton, p. 462-73, 1967.
12. Hottinger T, Virgilio C, Hall MN, Baller T, Wienken A: The role of trehalose synthesis for the aquisition of thermotolerance in yeast. J Biochem 21: 187-193, 1994.
13. Suomela H: Viral inactivation of blood and blood products: Global Blood Safety Initiative. WHO/LBS/Red Cross Blood Transfusion Service, Helsinki, Finland, pp. 2, 12-14, 1992.
14. Pasi KJ, Evan JA, Skidmose SJ, Hill FGH: Prevention of hepatitis C virus infection in haemophilias. Lancet 335: 1473-74, 1990.
15. Cirran OF: Understanding and increasing protein stability. Biochem Biophys Acta 1252: 1-14, 1995.
16. Hottiger T, Schmuty P, Wiemken A: Heat–induced accumulation and futile cycling of trehalose in *Saccharomyces cervisiae*. J Bacteriol 169: 5518-22, 1987.