Haematologica 1998; 83:305-311 original paper

Characterization and viral safety validation study of a pasteurized therapeutic concentrate of antithrombin III obtained through affinity chromatography

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Abstract

Background and Objective. **Antithrombin III (ATIII) concentrates are employed as therapy for congenital or acquired deficiencies. These concentrates are obtained from Cohn's fraction IV1. To improve yields, purity and safety, our group developed a procedure to obtain a pasteurized ATIII concentrate from the supernatant of Cohn's fraction II+III including a highly efficient heparin affinity chromatography purification and pasteurization as a viral inactivation step.**

Design and Methods. **Three steps of the manufacturing procedure (Cohn's fraction II + III precipitation, affinity chromatography and pasteurization) were selected to examine their efficacy in inactivating and/or removing the selected viruses.**

Results. **The industrial batches show a purity higher than 99% with approximately 95% native heparin binding ATIII. Only albumin and IgG could be detected at trace levels (0.07% and 0.16% of the total protein present, respectively). The specific activity of the product was approximately 6.65 IU/mg protein. Five viruses were spiked into the manufacturing starting materials and samples were collected at various points to determine the infection level of virus. The study showed a reduction factor (log 10) ≥ 11.7 for HIV-1; ≥ 8.1 for bovine herpes virus (analyzed as a model for herpes and hepatitis B viruses); ≥ 8.1 for bovine diarrhea virus (model for hepatitis C and G) and ≥ 6.0 for encephalomyocarditis virus (model for hepatitis A and other non-enveloped viruses).**

Interpretation and Conclusions. **No biochemical alterations of the ATIII were detected in the final product. A high viral elimination capacity of the production process was demonstrated. So far, more than 32 million units of ATIII have been transfused in the form of this therapeutic concentrate without any detected seroconversion.**

Key words: antithrombin III, pasteurization, therapeutic concentrate, affinity purification, viral safety validation

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Antithrombin III is a single-chain glycoprotein with an approximate molecular weight of 58 kDa which is synthesized in the liver and is present in plasma at a concentration of around 150 mg/L.[1] Its physiological function is to act as the main inhibitor of blood coagulation. Antithrombin III deficiency, whether acquired or congenital, may give rise to the formation of intravascular fibrin and thromboembolism. Deficiency is associated with various pathologies such as sepsis, traumas, liver failure and preeclampsia.[2-4]

Most commercially available therapeutic ATIII concentrates are obtained by affinity chromatography to heparin.[5] Capture and isolation of ATIII can be performed directly from plasma, although the fraction IV_1 obtained from the ethanol plasma fractionation process described by Cohn[6] is more often employed by the industry. Nevertheless, possibly due to the specific conditions under which the fraction IV_1 is precipitated, the content of ATIII found in this starting material is markedly lower than in the original plasma.[7] For this reason our development program was focused on elaborating a process that gave higher ATIII recovery than the one obtained from fraction IV_1. We also added one of the best-characterized virus inactivation steps: pasteurization.[8]

In order to ensure the maximum possible safety of the ATIII concentrate the viral inactivation and/or removal capacity was validated at three stages of the industrial process: fraction II+III precipitation of Cohn's fractionation with ethanol, an affinity chromatography step and pasteurization (60°C for 10 hours). To date, no specific cases of viral transmission[8] have been reported following the administration of ATIII. This study presents the *in vitro* characterization and the viral safety study of the ATIII concentrate obtained by this new procedure.

Materials and Methods

Starting plasma

The plasma for fractionation was obtained by plasmapheresis. European Pharmacopoeia requirement-compliant plasma (*Human Plasma for fractionation*, European Pharmacopoeia Monograph, 1993) was employed.

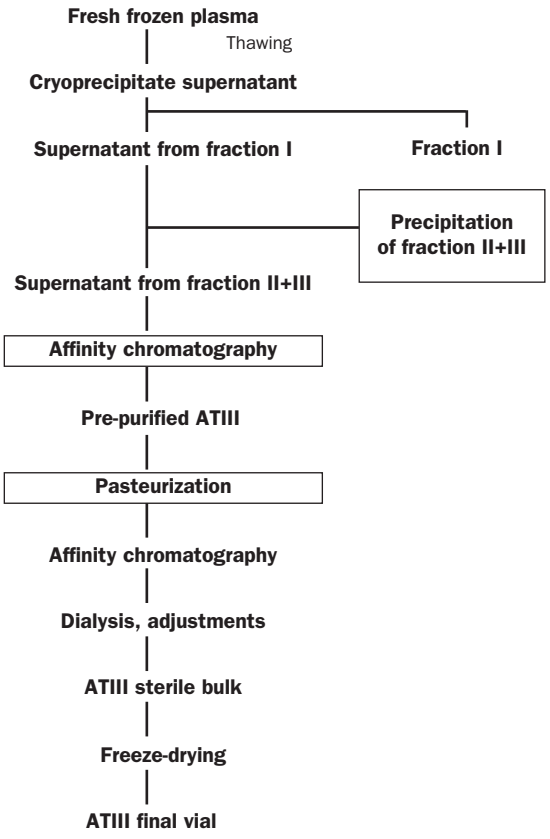

Figure 1. Flow diagram of the ATIII purification process. The steps selected for evaluation of viral safety are highlighted.

ATIII purification process

The starting material for the purification of antithrombin III was the supernatant from Cohn's II+III fraction.[6] Approximately 2000 litres of this fraction (equivalent to 1700 litres of plasma) was used for each batch.

The supernatant from Cohn's II+III fraction was initially purified by affinity chromatography (Figure 1) with a heparin-agarose gel. The dissociation between ATIII and heparin is performed using a saline buffer that allows the elution of the protein from the column. The eluate was concentrated by ultrafiltration and then pasteurized (60°C for 10 hours). After pasteurization a second affinity chromatography purification step was carried out followed once more by concentration through ultrafiltration. After concentration, adjustment of stabilizers (D-mannitol) and sterile filtration, glass vials were filled with 500 or 1000 international units (IU) of ATIII. Finally, the product was frozen and freeze-dried.

The relative protein content at the different steps of the process was determined using biuret reagent (supernatant fraction II+III) or by O.D. 280 nm [considering $E^{1\%}_{1cm}$ 7.9 (experimental results)].

Characterization of the final product

Purity was determined by cellulose acetate electrophoresis (Helena Laboratories). The molecular distribution of the protein was obtained by HPLC gel filtration with a TSK G3000SW column (Toyosoda, Japan, 7.5×600 mm).

For the stability study the vials were reconstituted and ATIII activity was determined using a thrombin-based chromogenic substrate assay (S-2238 Chromogenix), both at the beginning and after 4 hours of incubation in a temperature-controlled chamber at 25°C.

The heparin binding capacity of ATIII was determined by crossed immunoelectrophoresis in 1% agarose gels containing approximately 15 U of heparin/mL in the first dimension. The second dimension was performed in 1% agarose gel containing antibodies against ATIII supplied by Behringwerke Ag. The ATIII antigen content was determined in 1% agarose gel in the presence of the same antibodies using Laurell's quantitative immunoelectrophoresis method. The presence of possible accompanying proteins (albumin, IgG, fibrinogen, α_1-antitrypsin, haptoglobin, transferrin, α_1-acid glycoprotein and α_2-macroglobulin) was determined by nephelometry using a BN-100 Analyzer unit and specific antisera supplied by Behringwerke Ag.

Total protein was determined by Kjeldahl's method.

The determination of the approximate molecular weight of ATIII was carried out by SDS-PAGE with a PhastSystem unit on 10% to 15% gradient polyacrylamide gels using Pharmacia Biotech AB protein standards for the calibration of molecular weights. The staining procedure was carried out with silver nitrate using the Pharmacia PhastGel Silver Kit. The measurement of the approximate isoelectric point (pI) was carried out by isoelectric focusing (IEF) with a PhastSystem unit using polyacrylamide gels with linear pH gradients from 3 to 9 (PhastGel IEF 3-9, Pharmacia Biotech AB). The pI value was determined by interpolation to a protein standard of pI between 3-10. The staining procedure was performed using the Pharmacia Silver Kit.

Viral safety validation study

We elected to study three steps in the manufacturing process which are regarded as potentially effective in the inactivation or removal of viruses transmissible by blood-derived products: 1) precipitation of fraction II+III; 2) affinity chromatography; and 3) pasteurization (Figure 1).

Prior to the viral spiking studies these steps were downscaled and then validated at the reduced (laboratory) scale.

The performance of viral validation studies at manufacturing facilities is contrary to *Good Manufacturing Practice*. For this reason the study was carried out at Q-One Biotech Ltd., Scotland. The experimental val-

idation was conducted by adding viral inocula of known titre and then evaluating the residual infectivity in the fractions resulting from each step.

"Worst case" conditions

The study was performed under *worst case* conditions with process limits being set in order to provide the least advantageous scenario for viral inactivation or removal.

For the fraction II+III precipitation step the concentration of precipitating reagent and the contact time with the product was adjusted to the lowest limit admitted by production specifications in order to obtain the lowest possible removal of virus from the supernatant fraction (fraction used in the production of the antithrombin III concentrate).

For the affinity chromatography step the washing volume was adjusted to the lowest limit admitted whilst the elution volume was adjusted to the upper limit admitted by production specifications in order to obtain more viruses in the eluate.

For the pasteurization step temperature and heating time were adjusted to the lowest limits admitted thus providing the worst condition for viral inactivation through the effect of heat. Stabilizer (citrate) and protein concentration parameters were adjusted to their upper limit since this might provide a protective effect for the virus.

Viruses

Human immunodeficiency virus type 1 (HIV-1): enveloped virus, RNA of the *Retroviridae* family. This is a *relevant* virus and must therefore be included in viral removal/inactivation studies of blood derived products. The MRC RF strain supplied by MRC (UK) was used. HIV-1 virus inocula were prepared from Human H9 cells grown in RPMI 1640 medium containing 10% fetal calf serum (FCS), 0.02M L-glutamine plus 100 units of penicillin and 100 µg of streptomycin per mL. At the peak of syncitiation (2-3 days) the cells were harvested, clarified by centrifugation and passed into approximately four times the number of uninfected cells. The procedure was repeated as necessary until the peak cytopathic effect (CPE) was observed. The supernatant was then harvested, clarified by centrifugation, aliquoted and stored at or below –70°C. Samples containing $10^{8.2}$ 50% tissue culture infectious dose ($TCID_{50}$) were spiked to the starting sample in two independent procedures. C18166 cells grown in 96-well microtitre plates were infected with 50 µL of spiked sample dilutions using 10 replicates per dilution. Six days post-inoculation the wells were examined for cytopathic effect (CPE).

Bovine herpes virus type-1 (BHV-1): enveloped virus, DNA of the *Herpesviridae* family. This virus has been employed as a model for herpes and hepatitis B viruses. The LA strain (ATCC VR-188), supplied by ATCC (USA), was used. BHV-1 virus inocula were prepared from MDBK cells grown in Dulbecco's modified Eagles medium (DMEM) containing 1% FCS plus non-essential aminoacids (NEAA). Four or five days post-infection the cells were microscopically observed for CPE. When complete CPE was apparent in the culture the cells and medium were harvested and sonicated. The cell debris was removed by centrifugation and the resultant supernatant was filtered, aliquoted and stored at or below –70°C. Samples containing $10^{9.5}$ 50% tissue culture infectious dose ($TCID_{50}$) were spiked to the starting sample in two independent procedures. Confluent 24 well plates of MDBK cells were infected using eight replicates per dilution with 0.25 mL of spiked sample. Eight days post-inoculation the wells were examined for cytopathic effect (CPE).

Bovine viral diarrhea virus (BVDV): enveloped virus, RNA of the *Flaviviridae* family (also includes the hepatitis C virus). This is a virus with a moderate resistance to physicochemical agents which models hepatitis C and G viruses. The NADL strain (ATCC VR-534), supplied by ATCC (USA), was used. BVDV virus inocula were prepared from calf testes (Cte) cells grown in DMEM containing 1% FCS plus 1% NEAA. The cells were examined for CPE four to six days post-infection. When total CPE was apparent in the culture the cells and medium were harvested and rapidly frozen/thawed. The cell debris was removed by centrifugation and the resultant supernatant was filtered, aliquoted and stored at or below –70 °C. Samples containing $10^{6.5}$ 50% tissue culture infectious dose ($TCID_{50}$) were spiked to the starting sample in two independent procedures. Confluent 24 well plates of BT cells were infected using eight replicates per dilution with 0.25 mL of spiked sample dilution. Fourteen days post-inoculation the wells were examined for cytopathic effect (CPE).

Encephalomyocarditis virus (EMC): Non-enveloped virus. RNA, of the *Picornaviridae* family (which also includes the hepatitis A virus). This is a virus with a moderate-high resistance to physicochemical agents and therefore a good model for hepatitis A and other non-enveloped viruses. The ATCC VR-129B strain, supplied by ATCC (USA), was used. EMC virus inocula were prepared from Vero cells grown in Dulbecco's Modified Eagles Medium (DMEM) containing 1% FCS plus 1% non-essential aminoacids (NEAA). Twenty-four hours post-infection the cells were microscopically observed for cytopathic effect (CPE). When complete CPE was apparent in the culture, the cells and medium were harvested. The cell debris was removed by centrifugation and the resultant supernatant was filtered, aliquoted and stored at or below –70 °C.

Samples containing $10^{8.2}$ 50% tissue culture infectious dose ($TCID_{50}$) were spiked to the starting sample in two independent procedures; 24 well plates of confluent Vero cells were infected using eight replicates per dilution with 0.25 mL of spiked sample dilution. Eight days later the wells were examined for cytopathic effect (CPE).

Experimental protocol

The experimental design and validation of viral inactivation and/or elimination was performed according to the *European Guidelines issued by the Committee for Proprietary Medicinal Products* (CPMP)[9,10] and following Good Laboratory Practices duly certified by regulatory authorities.

Different preliminary tests were performed prior to the viral tests in order to guarantee the absence of cytopathic effect of the product on the susceptible cells.

The spiked samples were subsequently titrated in microtiter plates following thawing at 37 °C and dilution in DMEM (Dulbecco's modified Eagles) culture medium in 5-fold serial dilutions. All viruses were titrated by means of the $TCID_{50}$ infectivity test applying the Spearman-Karber method.[11] A minimum residual value based on the test detection limit and the volume of sample analyzed was determined in samples where no residual viral infectivity was detected. This value is obtained by approximation to a Poisson probability distribution.[12] In some experiments, analyzing higher sample volumes in order to increase the probability of detection of potential residual infectivity reduced the minimum detectable level.

As a measure of the virus removal/elimination capacity, the reduction factor is calculated as the log10 of the ratio between the amount of virus in the starting material and the amount of virus in the output material of the step being studied. The overall viral reduction capacity of the process can be estimated by adding the logarithmic reduction values of the individual steps.

Results

Purification results

Table 1 shows the results of the most representative parameters of the manufacturing process. The purification factor obtained was over 500 fold. The yield obtained in the ATIII sterile bulk is about 300 IU ATIII/l plasma.

Characterization

The results of the characterization are shown in Table 2.

The study of the heparin binding capacity of the ATIII preparations by crossed immunoelectrophoresis (Table 2, Figure 2) shows the presence of a small peak with low mobility, corresponding to ATIII with less heparin binding capacity, and a large peak with high mobility which corresponds to native ATIII and accounts for approximately 95% of the total protein.

No fibrinogen, α_1 antitrypsin, haptoglobin, transferrin, α_1 acid glycoprotein or α_2 macroglobulin was detected. Albumin and IgG were quantified as traces (0.06-0.11 μg/IU ATIII to 0.1-0.24 μg/IU ATIII, respectively).

The SDS-PAGE (Figure 3) showed, under reducing conditions, the presence of a single band with an approximate molecular weight of 67 kDa. Under non-reducing conditions the band presented a slight change of mobility resulting in an approximate molecular weight of 58 kDa.

The determination of the approximate isoelectric point (pI) of the ATIII (Figure 4) resulted in a pattern consisting of three bands, one major band with a pI of 4.75 and two minor ones with pIs of 4.82 and 4.90 respectively.

Table 1. Results of the purification process (n=4 lots).

Step	*% Protein (w/v)*	*Specific activity (IU/mg prot)*	*Yield (IU/L of plasma)*	*Purification factor*
*Plasma**	6.00	0.017	1000	1.00
Fraction II+III supernatant	3.22±0.37	0.021±0.001	814±115	1.24±0.09
First affinity chromatography eluate	0.64±0.15	7.50±1.29	394±73	441±38
Pasteurization	0.64±0.12	6.8±0.98	364±81	400±32
Second affinity chromatography eluate	0.59±0.2	9.82±1.46	320±79	577±64
ATIII sterile bulk	0.53±0.03	9.07±0.43	298±75	533±36

**Estimated theoretical data.*

Table 2. Biochemical characteristics of three batches of the ATIII concentrate. The stability was studied after reconstitution of the product (average±SD).

Purity	*Monomer*	*Stability 4h 25°C*	*ATIII with heparin-binding capacity*	*ATIII antigen IU ATIII:Ag*	*Specific activtiy IU ATIII / mg protein (n=15)*
Cellulose acetate (%)	*HPLC (%)*	*(%)*	*(%)*	*IU ATIII*	
99.7±0.1	97.5±1.27	100±1.91	94.8±0.65	0.93±0.01	6.65±0.44

Viral validation study

The results (Table 3) show that the fraction II+III precipitation step provides a slight but significant reduction in viral infectivity for HIV (2.4 log) and for BHV (1.5 log). The value for BHV is the worst result obtained in the two experiments carried out (1.5 log and 3.1 log, respectively).

The affinity chromatography reduced the viral infectivity of HIV-1 by 3.8 logs and of BVDV by 2.9 logs. Finally, pasteurization proved to be effective for all the viruses studied, either enveloped or non-enveloped (the latter being well known for their resistance to heat). Total inactivation was attained in all

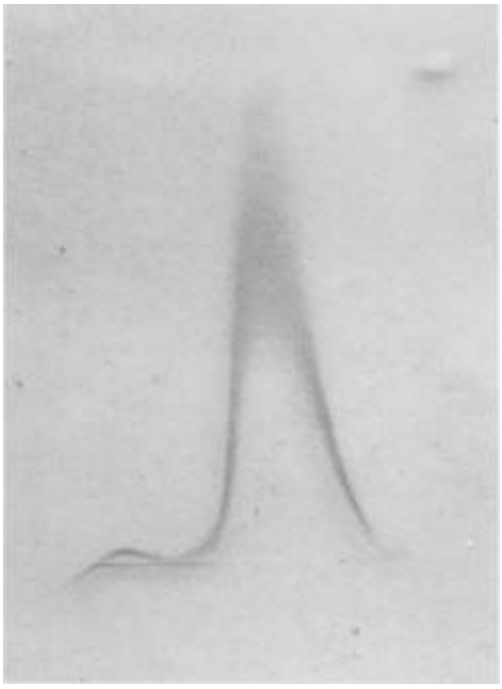

Figure 2. Heparin binding capacity of ATIII using crossed immunoelectrophoresis.

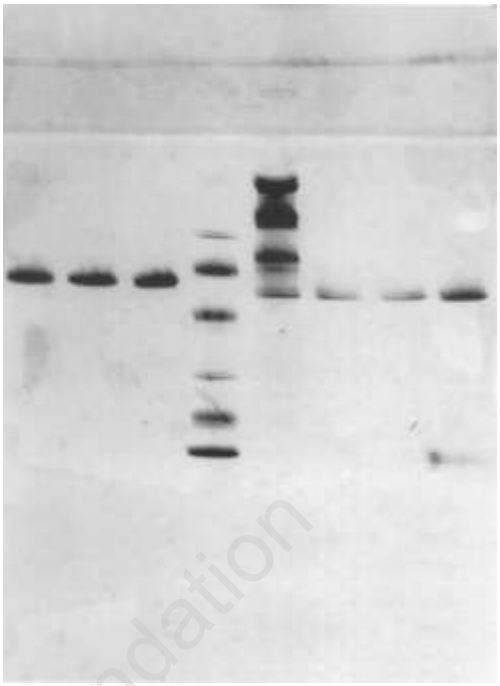

Figure 3. SDS-PAGE of three batches of ATIII concentrates under reducing (left) and non-reducing (right) conditions.

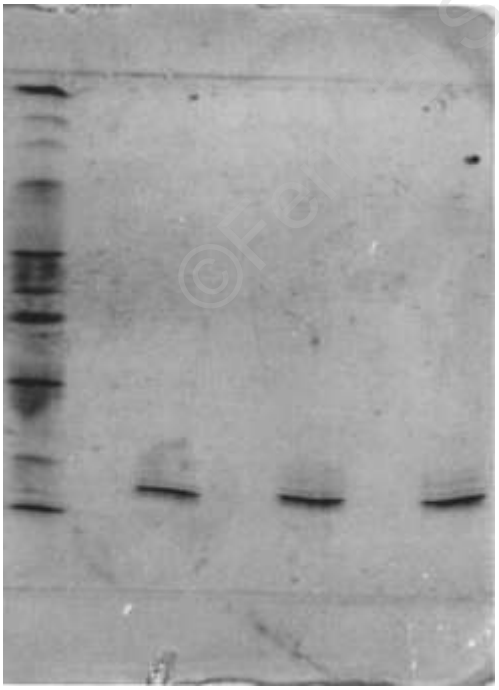

Figure 4. Isoelectric point (pI) obtained by IEF of three batches of ATIII concentrates.

cases as no residual infectivity was detected at the following sampling times: HIV (15 min), BHV (between 1 and 2.5 h post-treatment), BVDV (30 min). EMC showed a total absence of virus in one of the two experiments evaluated. The viral inactivation kinetics during pasteurization is shown in Figure 5.

Discussion

The use of a specifically designed chromatographic medium composed of a rigid matrix of agarose, highly replaced by heparin and with high dinamic capacity, has allowed the capture of ATIII and its isolation from fraction II+III supernatant with an improved yield. Recently, some authors[13] have identified a certain proportion of inactive conformations of ATIII (L-forms) formed during heat pasteurization. The introduction of a second affinity chromatography, following this viral inactivation step, leads to a reduction of these inactive forms of ATIII resulting in a highly purified final product (approximately 99%) which maintains its biochemical characteristics.

The stability study on the reconstituted product kept for 4 hours at 25±2°C in a temperature-controlled chamber shows a 100% recovery of ATIII activity compared to the initial value. This means that the stabilizers included in the final formulation preserve the protein from degradation without the need to add albumin as a stabilizer and so reduce the specific activity of the product.

Crossed immunoelectrophoresis reveals a percentage of heparin-binding ATIII (native ATIII) of about

Table 3. Logarithmic reduction factor of viral titre in different steps of the ATIII production process.

	Enveloped			Non enveloped
Target virus	HIV (RNA)	Herpes (DNA)	HCV (RNA)	HAV (RNA)
Virus studied	HIV-1	Bovine herpes (BHV)	Bovine diarrhea virus (BVDV)	Encephalomyocarditis virus (EMC)
Precipitation of fraction II+III	Exp.1: ≥ 2.1±0.2* Exp.2: 2.7±0.4	Exp.1: 1.5±0.3 Exp.2: 3.1±0.3	Exp.1: < 1.0 Exp.2: < 1.0	Exp.1: < 1.0 Exp.2: 1.3±0.4
Representative reduction factor	2.4±0.4*	1.5±0.3*#	< 1.0	<1.0
Affinity chromatography	Exp. 1: ≥3.5±0.2* Exp. 2: 4.0±0.2	Exp.1: <1.0 Exp.2: 2.6±0.2	Exp.1: >4.3±0.2* Exp.2: 2.9±0.2	Exp.1: <1.0 Exp.2: 2.4±0.4
Representative reduction factor	3.8±0.3*	< 1.0#	2.9±0.2	< 1.0#
Pasteurization (60 °C, 10h)	Exp.1: ≥5.6±0.2* Exp.2: ≥5.4±0.2*	Exp.1: >7.7±0.2* Exp.2: >6.6±0.2*	Exp. 1: ≥ 4.8±0.2* Exp. 2: ≥5.2±0.4*	Exp. 1: ≥ 6.1±0.0 Exp. 2: 6.0±0.3
Representative reduction factor	≥5.5 +0.3* (15 min)	≥ 6.6 +0.2* (1 to 2.5 h)	≥ 5.0 +0.4* (30 min)	6.0 +0.3* (1 Run: 2.5 h)
Overall reduction factor°	≥ 11.7	≥ 8.1	≥ 7.9	6.0

**No residual virus detected. The representative reduction factors express the average of the two experiments performed.*
#Where the difference in value of the reduction factors between the two experiments is higher than 1 log, the worst result is shown).
°Only the logarithmic reduction factors equal to or greater than 1 log were taken into consideration.

95% from total protein. This percentage is markedly higher than that permitted by the European Pharmacopoeia 1997 for therapeutical concentrates of ATIII (60%). It has been suggested that denatured ATIII may be harmful to patients receiving it as it may cause the release of cytokines.[14] The ratio of ATIII:Ag/ATIII amydolitic activity which is close to one is evidence that there is no denaturation of the final product during the manufacturing process. The average specific activity obtained when analyzing 15 batches is 6.65±0.44 IUATIII/mg protein. This value accords with the virtual absence of detectable accompanying proteins. The three bands of ATIII, obtained at pI values of 4.75, 4.82 and 4.90 respectively, are within the pI range (from 4.7 to 5.2) found in ATIII in human serum.[15]

All biological products have a potential viral transmission risk. Since the introduction of screening tests (HBsAg, anti-HCV, HIV Ag p24, Anti-HIV-1 and 2 and ALT) and recently, of genomic amplification

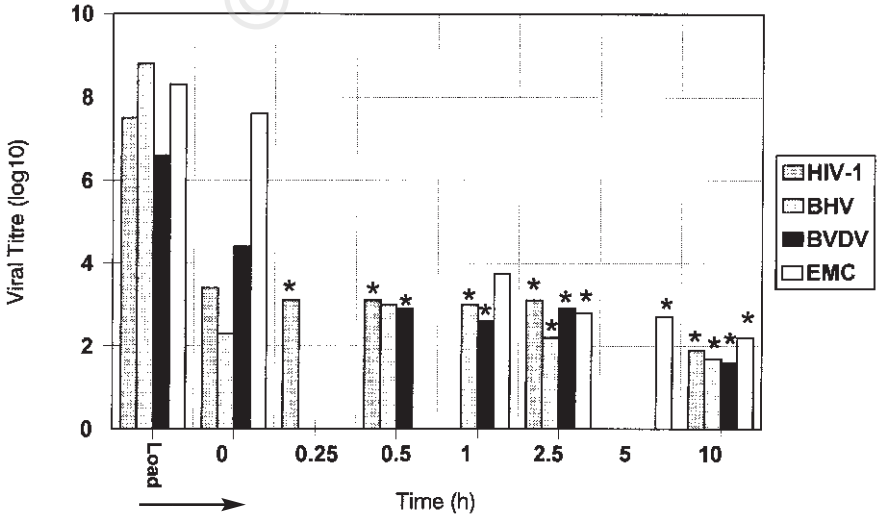

Figure 5. Inactivation kinetics of the HIV, BHV, BVDV, EMC virus by heating at 60°C for 10 hours.

Note.
- *At 0.25h BHV, BVDV and EMC were not studied. At 5h HIV, BHV and BVDV were not studied.*
- *X axis values are not represented in an arithmetical scale.*
- *Asterisk indicates that no residual virus was detected in two runs and the minimum detectable level is shown. For EMC no residual virus was detected in one of the two experiments evaluated. The arrow indicates the period of preheating time required to reach 59.6°C (< 30 minutes).*

techniques, it has become possible to reduce considerably the potential viral load in plasma for fractionation. Additionally, specific inactivation steps have been introduced to increase product safety. Our study illustrates the inactivation capacity of the Antithrombin III production process, particularly that of pasteurization (10h at 60°C) which yields a high degree of viral inactivation for all the viruses studied whether enveloped (less heat resistant) or non-enveloped (more heat resistant).

The rapid total inactivation of HIV (understood as the absence of detectable residual infectivity) after only 15 minutes of the heating process (reduction factor ≥ 5.5 log) is particularly notable. The kinetic analysis of BHV shows total inactivation of viral infectivity 2.5 hours after the beginning of treatment providing a reduction factor of ≥ 6.6 log. The results of both studies were consistent. BVDV, possibly the best hepatitis C model, also showed full inactivation (reduction factor ≥ 5.0 log) 30 minutes after reaching 59.6°C.

Finally, EMC, studied as a model for hepatitis A virus, known for its high heat-resistance and stability,[16] yielded a viral inactivation of 6.0 log during pasteurization. Minimal residual infectivity was detected only in one experiment. Total viral inactivation was attained in the other experiment 2.5 hours after reaching 59.6°C.

The other steps studied (affinity chromatography and the precipitation of the fraction II+III) provide an additional degree of removal of the viruses studied, thereby rendering this ATIII manufacturing process more effective in terms of viral safety.

The production process includes the additional safety measure of the routine analysis of hepatitis C virus by PCR in pilot pools made up of the sampling tubes of plasma to be fractionated. Furthermore, each batch of finished product of ATIII concentrates is submitted to routine PCR analysis for HCV. Between September 1995 and July 1997 (date of preparation of this manuscript), 142 batches of product have been analyzed with no positive result detected.

Contributions and Acknowledgments

HB collaborated in the viral validation studies design. She was responsible for the coordination and supervision of these studies results and participated in their analysis and intrepretation. She wrote the paper in cooperation with MG. MG was responsible for the biochemical analysis of the purification process and final product. JF carried out the purification processes (industrial and pilot scale). PR collaborated in the viral validation studies design. He was responsible of the purification process. MM formulated the design of the characterization study and participated both in the viral validation studies design and in the final revision of the paper. EW was responsible for viral titrations. FV collaborated in viral validation studies design. All authors contributed to the analysis, revision and approval of the paper. The authors wish to thank Mr. Mark McDonnell for his assistance in reviewing the manuscript.

Disclosures

Conflict of interest: Institute Grifols and Q-one Biotech Ltd produce antithrombin III for commercial purposes.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received April 29, 1997; accepted January 12, 1997.

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