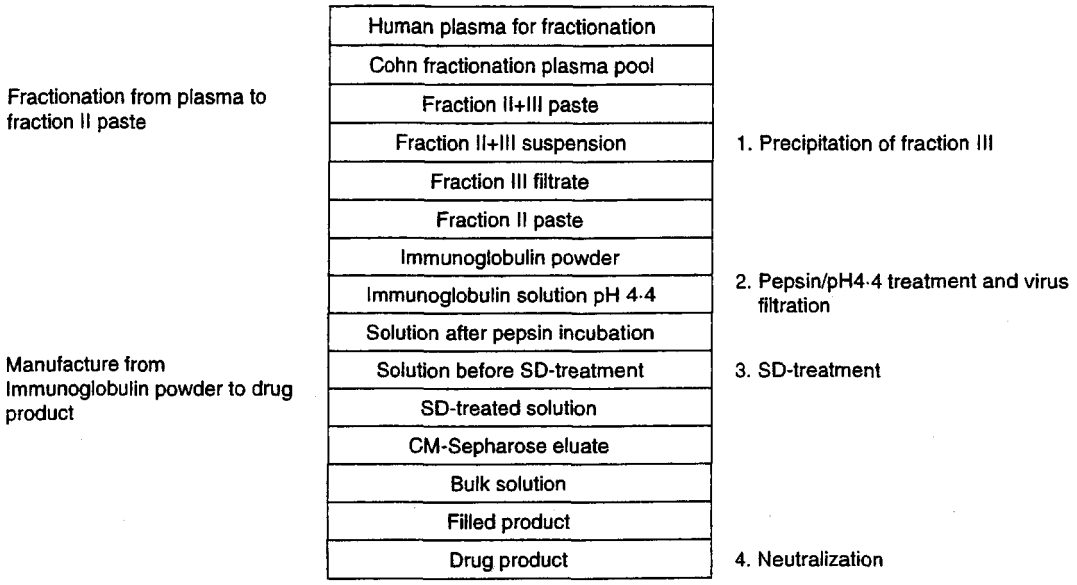

Fig. 1 Flow chart of the manufacturing process of Nanogam®, starting from human plasma. Human plasma for fractionation is processed to immunoglobulin powder; the process intermediate is subjected to the 15-nm filtration step combined with pepsin treatment at pH 4·4 (pH 4·4/15NF) and solvent–detergent (SD) step. Subsequently, a polishing step is applied and the resulting product is filled out as a liquid immunoglobulin product.

Introduction

During the manufacture of human plasma derivatives, a series of complementary measures are undertaken to prevent viral transmission to the recipient. These measures include the selection of voluntary and non-remunerated donors, the exclusion of donors at risk, the screening of donations for a range of relevant blood-borne viruses and demonstration of adequate viral reduction by effective inactivation or removal steps in the manufacturing process. These measures have contributed significantly to the safety of plasma derivatives, and the residual risk for human immunodeficiency virus (HIV) or hepatitis C virus (HCV) transmission in blood transfusion is currently close to one in a million [1,2].

Human plasma-derived immunoglobulin products are typically manufactured by the Cohn ethanol fractionation process and frequently include a low-pH treatment step [3,4]. The efficacy of this step to reduce the amount of viruses is limited for non-lipid-enveloped (NLE) viruses and usually does not meet the criteria for an effective step [4–6]. Precipitation of Cohn fraction III typically results in a 3–4 $\log_{10}$ reduction for the lipid-enveloped (LE) and NLE viruses, whereas a pH 4·4 treatment step typically shows variable inactivation for LE viruses [3,6,7] and very low inactivation of NLE viruses [3]. Finally, for immunoglobulin products, a contributing virus-reduction effect of neutralizing antibodies directed against parvovirus B19 (B19) and hepatitis A virus (HAV) is described [8–10].

Although immunoglobulin products have an excellent safety record, European regulators encourage implementation of additional effective virus-reducing steps into manufacturing processes to further ensure the safety of these products. In general, a manufacturing process should encompass validated effective steps for the inactivation/removal of viruses. In order to achieve this, it will be desirable in many cases to incorporate two distinct effective steps that complement each other in their mode of action, such that any virus surviving the first step would be effectively inactivated/removed by the second. At least one of the steps should be effective against non-enveloped viruses. Whether a virus-removal/inactivation step is classified to be effective, moderately effective, or ineffective is judged on a series of considerations, including the validity of virus and scale-down systems used, the log-reduction factors demonstrated, assessment of the mechanism and robustness of viral reduction [11–13].

In the present study we evaluated the virus-reducing capacity of a new manufacturing process for a liquid immunoglobulin product, Nanogam®, including two effective virus-reducing steps (Fig. 1). These steps are a combined pepsin/pH 4·4 treatment and 15-nm filtration step (pH 4·4/15NF step) and a solvent–detergent (SD) treatment step. Nanofiltration has successfully been implemented in many manufacturing processes for various plasma derivatives in recent years [14–16], primarily because nanofiltration can effectively remove NLE viruses, with relatively little impact on the product. The most frequently used filters have a mean pore size of 15–20 nm. These filters are suitable for the removal of small NLE viruses, such as B19 and HAV, and are particularly useful for filtering relatively small therapeutic proteins, such as factor IX. For larger proteins (e.g. factor VIII

Table 1 Properties of the selected viruses

Virus group	Virus	Virus family	Genome	Size (nm)	Model virus for
Lipid-enveloped (LE)	BVDV	Flavi	ssRNA	37–50	Hepatitis C virus
	HIV	Retro	2 ssRNA	100	Relevant virus
	PRV	Herpes	dsDNA	100–200	Large LE dsDNA viruses
Non-lipid-enveloped (NLE)	B19	Parvo	ssDNA	18–26	Relevant virus
	BPV	Parvo	ssDNA	18–26	Human parvovirus B19
	CPV	Parvo	ssDNA	18–26	Human parvovirus B19
	EMC	Picorna	ssRNA	22–30	Hepatitis A virus
	HAV	Picorna	ssRNA	22–30	Relevant virus

B19, parvovirus B19; BPV, bovine parvovirus; BVDV, bovine viral diarrhoea virus; CPV, canine parvovirus; ds, double-strand; EMC, encephalomyocarditis virus; HAV, hepatitis A virus; HIV, human immunodeficiency virus; PRV, pseudorabies virus; ss, single strand.

and immunoglobulin) usually filters with a larger mean pore size, of 35–50 nm, are used [17,18]. By carefully optimizing the conditions of the nanofiltration step in the Nanogam® process it became possible to pass an immunoglobulin product through a 15-nm filter with a good yield (at least 95%) and thereby benefit from the virus-removal capacity of this step for smaller NLE viruses. An SD treatment step was also included in the manufacturing process. SD treatment has been used in many manufacturing processes for plasma derivatives and other biologicals and is the most effective virus-inactivation step for LE viruses. Evaluation of the effectiveness of virus-reducing steps also includes the demonstration of robustness. The concept of robustness was put forward by the European regulators in the mid-1990s to provide information on the influence of various process parameters on the virus-reducing capacity of a virus-reducing step. Previously, we presented the use of fractional factorial designs to demonstrate the robustness of various process steps, including nanofiltration and SD steps [19]. The concept of such experimental designs is not to vary one factor at a time, but to vary all process parameters simultaneously, but in a structured manner. Such experimental designs are efficient and provide statistically sound data. Finally, we studied the contribution of the existing steps – precipitation of Cohn fraction III and immune neutralization – for overall virus-reduction capacity.

In this study, the behaviour of B19 vs. CPV during the precipitation of fraction III and the pH 4·4/15N step was investigated. The rationale for comparing the behaviour of B19 and CPV in these two steps is based on a number of publications. Blumel *et al.* [20] have shown that B19 is relatively sensitive to heat treatment when compared with porcine parvovirus. This observation was confirmed by Yunoki *et al.* [21]; in a comparative study, B19 was inactivated much faster than CPV by heat treatment. Besides the different behaviour after heat treatment, Boschetti *et al.* [22] also found that the susceptibility of B19 and minute virus of mice (MVM) to inactivation by treatment at low pH was different.

Materials and methods

Viruses and cells

For this study, a range of LE and NLE viruses were used and these viruses were propagated in suitable cell lines (Table 1). Bovine viral diarrhoea virus (BVDV), strain NADL (VR-534; ATCC, Rockville, MD) was cultured on MDBK cells (CCL-22; ATCC) and titrated on EBTr cells (ID-Lelystad, Lelystad, the Netherlands). Bovine parvovirus (BPV), strain Haden (Biotest Pharma, Dreieich, Germany), was cultured on MDBK cells (CCL-22; ATCC) and titrated on EBTr cells. Canine parvovirus (CPV), strain 780916 (State University Rotterdam, Rotterdam, the Netherlands), was cultured and titrated on A72 cells (State University Rotterdam). Encephalomyocarditis virus (EMC), strain EMC (VR-129B; ATCC), was cultured and titrated on VERO cells (CCL-81; ATCC). HAV, strain HM175/18F (Organon, Boxtel, the Netherlands), was cultured and titrated on BSC-1 cells (Organon). HIV, strain HTLV-IIIB (National Cancer Institute, Bethesda, MD), was cultured on H9 cells (National Cancer Institute) and titrated on MT2 cells (Wellcome, Beckenham, Kent, UK). Pseudorabies virus (PRV), strain Aujeszki Bartha K61 (Duphar, Weesp, the Netherlands), was cultured and titrated on PD5 swine kidney cells (Duphar).

Test for cytotoxicity

Prior to initiation of the cytotoxicity assay, cells were suspended in 4·0 ml of culture medium, transferred into 25-cm^2 tissue-culture flasks and incubated for 1 day at 37 °C. Subsequently, threefold serial dilutions of the test sample were prepared in culture medium and tested in 0·5-ml volumes on cells in duplicate. Unexposed cells were used as control cultures.

Subsequently, all cell cultures were incubated at 37 °C for the period required for the respective virus systems. The cytotoxicity observed is expressed as a lowest dilution of the test sample that was free of any cytotoxic effects.

Test for stop-and-storage conditions and interference

A virus ampoule was thawed and diluted in culture medium to ≈ $10^{5.3}$ tissue culture infectious dose 50% ($TCID_{50}$) per ml (virus inoculum). To test the efficacy of the stop condition, the selected dilution of a test sample was prepared in culture medium, and 9·5 ml of this dilution was spiked with 0·5 ml of virus and then incubated for 30 min at room temperature. After incubation, the infectivity of the virus inoculum and the test sample were measured directly to determine the efficacy of the stop condition and lack of interference. The virus inoculum was titrated with the standard $TCID_{50}$ assay (dilution in culture medium), whereas the test sample was titrated in a modified $TCID_{50}$ assay (dilution in prediluted test sample). To determine whether the selected dilution of the test sample also provided an effective storage condition, samples of the virus inoculum and spiked-and-incubated test sample were also frozen and stored for at least 7 days, and subsequently tested for infectivity. If the selected dilution of test sample provides an effective stop or storage condition, and does not cause interference, no significant loss of infectivity is expected (clearance factor < 1 $\log_{10}$).

Neutralization assays

To demonstrate the B19- and HAV-neutralizing activity in the final Nanogam® product, we used an identical experimental setup as used for testing the stop condition and interference, measuring the reduction of virus titre after incubation at room temperature for 30 min. BPV was used to measure neutralizing activity against B19, in view of the fact that B19 antibodies can cross-neutralize BPV [18,23].

Virus assays

Infectivity was measured in validated $TCID_{50}$ assays and bulk culture tests. For $TCID_{50}$ assays, threefold serial dilutions of samples were prepared in culture media, and 50-µl (or 0·5 ml for HIV) volumes were tested in eight replicates. To detect small amounts of virus, up to 60 ml of prediluted sample was tested in duplicate bulk culture tests using 25- and 175-cm^2 flasks. BVDV, BPV, CPV, EMC and PRV cultures were inspected microscopically for cytopathic effects at 6, 14, 7, 6 and 5 days postinfection (d.p.i.), respectively. Supernatants of HAV cultures were harvested after 14 d.p.i. and subsequently tested in an HAV enzyme-linked immunosorbent assay (ELISA). HIV cultures were inspected microscopically twice a week for the formation of syncytia until 21 d.p.i. Virus titres were calculated by the Spearman–Kärber method, and expressed as $TCID_{50}$/ml. If all cultures were negative, the titre ($TCID_{50}$/ml) was considered to be less than 1 ÷ total test volume (ml). Reduction factors (RF) were calculated by the following formula:

RF = $\log_{10}$(total amount of virus spiked as derived from the reference sample ÷ total amount of virus recovered from the treated sample).

Clearance factors (CF) were calculated by the following formula:

CF = $\log_{10}$(total amount of virus spiked as derived from the virus-stock sample ÷ total amount of virus recovered from the treated sample).

B19

B19-containing plasma (VP2-IgM and VP2-IgG antibody negative) was obtained from a highly viraemic blood donor during the window-phase of the infection.

Nucleic acid was extracted in 50 µl using automated sillica extraction (NucliSens; Biomerieux, Boxtel, the Netherlands). The amount of B19 DNA was quantified with a commercially available internal control (IC)-controlled real-time polymerase chain reaction (PCR) assay (LightCycler; Roche, Mannheim, Germany) [24] using 5 µl of eluate as the input for amplification. Different starting materials were used in this study. To study possible matrix effects, each starting material was mixed with 5 µl of IC. The nucleic acid of the starting material was isolated and B19 DNA IC molecules were amplified. For comparison, the same experiment was performed using normal human plasma (NHP) as a control. NHP does not interfere with the assay [24]. Starting material was considered not to interfere with the assay when the IC signal was positive and the difference between the crossing points of the starting material and the control was less than four cycles.

Downscaling

Design, validation and performance of the downscaled version of the manufacturing process steps examined (pH 4·4/15NF, SD step and precipitation of fraction III) were performed according to the requirements described in the prevailing guidelines [12]. The values of relevant parameters for these steps performed at full-scale were compared with those obtained in the downscaled steps. The differences observed were within set specifications. To ensure that identical starting materials were used for downscaled studies, all starting materials used for these studies were obtained from process intermediates of the full-scale production process.

For the downscaled pH 4·4/15NF step, a 0·01-m^2 Planova 15N filter was used, whereas for the full-scale step, 6 × 1-m^2 filters in parallel were used (scale 1 : 600). The Planova 15N virus filter is a hollow-fibre filter with a mean pore size and range of 15 ± 2 nm. The dimensions of the hollow fibres of the 0·01-m^2 and the 1-m^2 filters are the same, except that the length of the fibre in the 0·01-m^2 filters is 2·5 times shorter than the length of the 1-m^2 filter. Therefore, the tangential flows at downscale and full-scale were normalized and set at an identical value, expressed as l/cm fibre/h. The filtrate flows at both downscale and full scale were 4 l/m^2/h at t = 0 h and decreased linearly to 1·5 l/m^2/h at t = 24 h. The protein yield into the filtrate was 92–97% (n = 3) for downscale, but 97–100% (n = 5) for full scale.

The downscaled pH 4·4/15NF step was performed with 540 ml of starting material (50 g/l protein, 0·3% NaCl), from Sanquin (SQ) and the Finnish Red Cross (FRC). This material was adjusted to pH 4·4 and subsequently pepsin (1 : 10 000, w/w of protein) was added. After prefiltration with a 0·1-µm filter, the material was incubated at 35 °C for 4 h. After this incubation, the material was passed over a Planova 15N filter (0·01-m^2, tangential mode, pressure 0·8 bar, cross-flow 8 ml/min) while maintaining the temperature at 35 °C. The total incubation time of the material at 35 °C was 42 h, including the nanofiltration step.

The SD step (scale 1 : 20 000) was performed using 0·3% tri (n-butyl) phosphate (TNBP), 1% Tween 80, at pH 5·0 ± 0·1, and incubation at 26 °C for 6 h. Briefly, 25 ml of starting material was transferred into a thermostatic reaction container and, after temperature adjustment, the SD chemicals were added while mixing well.

The downscaled precipitation of the fraction III step (scale 1 : 13 000) was performed using 150 g of fraction II+III solution to which 96% ethanol was slowly added during a time-period of ≥ 12 h to a final concentration of 12%. During the addition of ethanol, the temperature was decreased from 0·25 °C to −3 °C and, after the addition of ethanol, the mixing was continued overnight. Subsequently, extra filter aid (final concentration 2%) was added and a filtration procedure (filtration area 9 cm^2 with a cellulose filter sheet) was applied to obtain the filtrate in two subsequent fractions and a resuspended paste III.

Results

Selection of viruses

The selection of viruses (Table 1) for the steps studied was based on the European guidelines for virus-validation studies of plasma derivatives [11–13]. The LE viruses HIV, BVDV and PRV were selected; HIV as a relevant blood-borne virus, BVDV as a specific model virus for hepatitis C virus, and PRV as a general model virus for LE DNA viruses, such as hepatitis B virus. The NLE virus, B19, was selected as a relevant blood-borne virus, and CPV and EMC were selected as specific model viruses for human parvovirus B19 and HAV, respectively. EMC was used, rather than HAV, as significant amounts of anti-HAV-neutralizing antibodies are present in Nanogam®. However, for demonstrating the virus-neutralizing activity of Nanogam®, HAV was used together with BPV, which is cross-neutralized by anti-B19 antibodies.

Combined pH 4·4 treatment and the 15-nm filtration step

The virus-reducing capacity of the pH 4·4/15NF step was studied for B19, CPV and EMC, and for BVDV, HIV and PRV. For the two groups of viruses, different experimental designs were used to take account of the size of the virion compared with the mean pore size of the filter used and the susceptibility of the viruses to pH 4·4 treatment. As the NLE viruses are relatively resistant to pH 4·4 treatment, removal of these viruses will be the dominating mechanism of virus reduction. Therefore, an experimental design was used to study the filtration process in detail, collecting samples from the 0·1-µm prefiltrate, from four sequential filtrate fractions and from the wash, whereas a limited set of samples was collected to demonstrate inactivation as a result of pH 4·4 treatment. The fractions were collected after the first and second 10%, the subsequent 60% and after the final 20% of the total filtrate. The rationale for selection of these fractions was that for tangential filtration, breakthrough is expected early during filtration while building up a protein layer. The retentate was collected to enable determination of mass balance.

For CPV, significant (≈ 4 $\log_{10}$), but incomplete, removal was found for the pH 4·4/15NF step (Table 2, normal run). In all filtrate fractions, break-through of virus was observed. There was no significant reduction after 0·1-µm prefiltration, suggesting that aggregation of virus was limited. The bench control (42 h at 35 °C) showed only limited inactivation during the process, and a similar reduction was found for the retentate sample, demonstrating that the main mechanism of virus reduction is removal. For EMC, similar results were found, except that this virus was removed completely by the pH 4·4/15NF step. For B19, complete removal was found in all fractions and no reduction was found in the 0·1-µm prefiltration, in the bench control or in the retentate sample.

As the LE viruses, BVDV and HIV, are relatively susceptible to pH 4·4 treatment and represent relatively large virion sizes compared with the mean pore size of the 15-nm filter, we anticipated finding complete removal of these viruses by the filtration process. Therefore, an experimental design was selected consisting of sampling only 0·1-µm prefiltrate and pooled filtrate-and-wash samples, whereas multiple samples were taken to demonstrate the inactivation kinetics caused by the pH 4·4 treatment.

Table 2 A 15-nm filtration step combined with pepsin treatment at pH 4·4 (pH 4·4/15NF) for canine parvovirus (CPV), parvovirus B19 (B19) and encephalomyocarditis virus (EMC) (reduction factor values are shown)

Sample	CPV (normal run)	CPV (worst-case run)	B19 (normal run)	EMC (normal run)
Bench control (42 h at 35 °C)	0·8	1·0	0·0	1·4
Filtrate 0·1 µm	0·0	−0·1	0·0	0·7
Filtrate fraction 0–10%	> 3·4 but ≤ 4·2	> 4·6	> 6·1	> 5·8
Filtrate fraction 10–20%	> 4·2 but ≤ 5·2	> 3·6 but ≤ 4·3	> 6·1	> 5·8
Filtrate fraction 20–80%	> 4·3 but ≤ 5·3	> 3·6 but ≤ 4·3	> 6·1	> 5·8
Filtrate fraction 80–100%	> 4·2 but ≤ 5·6	> 3·6 but ≤ 4·3	> 6·1	> 5·8
Retentate	1·8	1·1	0·1	1·4

The pH 4·4/15NF treatment was tested for removal of CPV, B19 and EMC. The results of normal runs (standard conditions) are shown; for CPV the results of the worst-case run are also shown. The total amount of spiked virus was 7·6 $\log_{10}$ (CPV normal run), 6·8 $\log_{10}$ (CPV worst-case run), 10·6 $\log_{10}$ (B19) and 7·4 $\log_{10}$ (EMC), respectively.

For both BVDV and HIV, complete reduction was found for the pH 4·4/15NF step (Table 3). After 0·1-µm prefiltration, no reduction for BVDV, and significant reduction for HIV, was observed. The bench control samples collected after 42 h of treatment showed significant, but incomplete, inactivation for BVDV, and complete inactivation for HIV, demonstrating that the mechanisms of virus reduction for these viruses are both removal by nanofiltration and inactivation by pH 4·4 treatment. The inactivation kinetics for the pH 4·4/15NF step for BVDV and HIV is further shown in Fig. 2(a).

As PRV is highly susceptible to inactivation by pH 4·4 treatment, complete inactivation was expected within 42 h of treatment. Therefore, the experimental design for PRV was limited to collecting only samples for demonstrating the inactivation kinetics of PRV by pH 4·4 treatment. Finally, because PRV is a relatively large virion compared with the mean pore size of the 0·1-µm prefilter, the 0·1-µm prefiltrate step was omitted in the experimental design. For PRV, complete inactivation was found to the limit of detection after 4 h of treatment (Fig. 2b).

Table 3 A 15-nm filtration step combined with pepsin treatment at pH 4·4 (pH 4·4/15NF) for bovine viral diarrhoea virus (BVDV) and human immunodeficiency virus (HIV) (reduction factor values are shown)

	BVDV		HIV	
Sample	Run A	Run B	Run A	Run B
Bench control (42 h at 35 °C)	3·0	≥ 3·3 but ≤ 5·2	> 6·1	> 5·6
Filtrate 0·1 µm	−0·4	−0·2	3·0	2·2
Filtrate	> 6·3	> 6·4	> 6·0	> 5·4

The pH 4·4/15NF treatment was tested for removal of BVDV and HIV in duplicate runs (A and B). The total amount of spiked virus was 8·5 (run A) and 8·6 (run B) $\log_{10}$ (BVDV runs) and 8·2 (run A) and 7·7 (run B) $\log_{10}$ (HIV runs), respectively.

To further investigate the robustness of the pH 4·4/15NF step, seven process parameters were selected and studied using a two-level factorial design [25]. The basis of experimental design is to deliberately introduce variation in all process parameters simultaneously, but in a structured manner. The method provides statistically reliable information and enables interactions between process parameters to be studied. Using experimental design is fundamentally different from the classical 'change-one-factor-at-a-time' strategy, where only one process parameter is varied, while all other process parameters are fixed. We selected an experimental design at two levels (i.e. testing only the upper and lower limits for every process parameter). For example, in Table 4 the standard pressure for filtration was 0·8 bar and the chosen upper and lower limits were 1·0 bar (+) and 0·6 bar (−). In the design matrix the values were changed in the following manner, −, −, +, +, −, −, +, + for runs 3–10, respectively. Runs 1 and 2 were performed under standard process conditions and are included to assist analysis of the data.

The selected process parameters were: source of starting material; pressure; temperature; protein concentration; pH; cross-flow; and ionic strength. The levels for the selected parameters were set just beyond the limits defined for the process step. For the robustness study, only CPV (i.e. the worst-case virus for this step) was used. The titres from this robustness study were calculated only with the most probable number (MPN) method [26] as the titres obtained from the Spearman-Kärber method resulted in censored data (the RF value is between an upper and a lower limit, e.g. > 4·3 but ≤ 5·3). Subsequently, the RF values obtained from the MPN method were modelled using linear regression analysis. This modelling showed that pH is the most dominant factor, whereas transmembrane pressure, cross-flow and source material did not influence the results of Planova filtration.

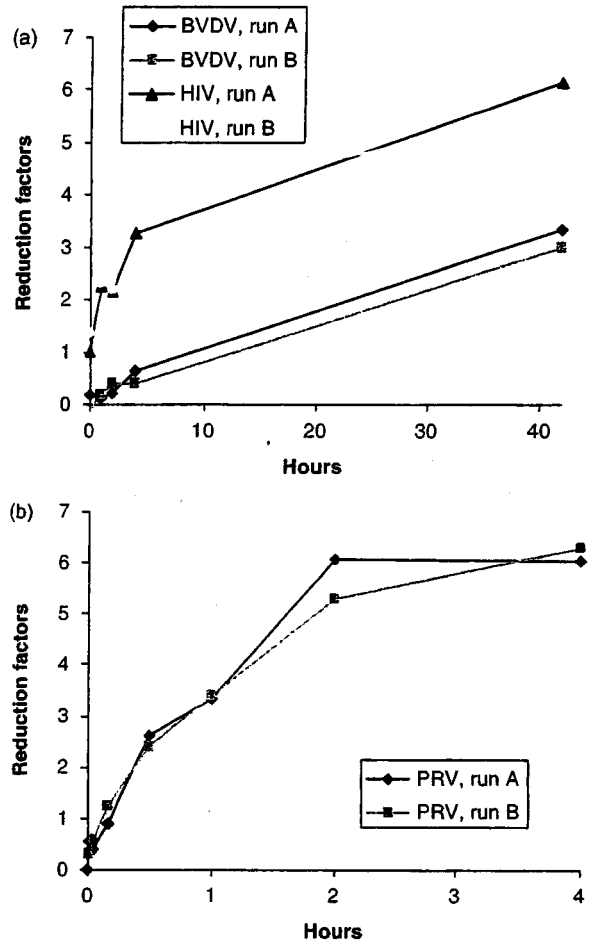

Fig. 2 Inactivation kinetics of bovine viral diarrhoea virus (BVDV), human immunodeficiency virus (HIV) and pseudorabies virus (PRV) as a result of pH 4·4 treatment. (a) Virus-inactivation kinetics for BVDV and HIV were studied until 42 h of treatment. (b) Virus-inactivation kinetics for PRV were studied until 4 h of treatment. Filled marker, the exact value of the calculated reduction factor (RF) is indicated; open marker, the RF was equal to or higher than the indicated value. The total amount of spiked virus for BVDV was 8·6 and 8·5 $\log_{10}$ for duplicate runs A and B; the total amount of spiked virus for HIV was 8·2 and 7·7 $\log_{10}$ for duplicate runs A and B; and the total amount of spiked virus for PRV was 8·1 and 8·3 $\log_{10}$ for duplicate runs A and B, respectively.

Protein concentration, temperature and ionic strength had only a minor effect on virus removal by Planova filtration (Table 4). To confirm the findings of the robustness study, the deduced worst-case condition (temperature 38 °C; protein concentration 45 g/l; pH 4·32; ionic strength 0·2%) was studied with CPV, and a reduction factor of > 4·6 $\log_{10}$ was found for the filtrate fraction 0–10%, and > 3·6 but ≤ 4·3 $\log_{10}$ for the filtrate fractions 10–100% (Table 2, worst-case run). Therefore, even under worst-case conditions, a reduction of ≈ 4 $\log_{10}$ can be demonstrated using the worst-case virus.

SD treatment

Assuming extremely fast inactivation kinetics, a study was performed collecting samples after 10 min of SD treatment of BVDV, HIV and PRV, testing the standard SD and an SD diluted 1 : 3 (Fig. 3). For the standard SD concentration, the clearance factors after 10 min were > 6·3, 5·9 and > 6·9 $\log_{10}$ for BVDV, HIV and PRV, respectively. For the SD diluted 1 : 3, slightly slower inactivation kinetics were observed for all viruses. Complete inactivation was found for BVDV and HIV after 10 min of treatment, whereas inactivation for PRV was not complete (but more than 5·0 $\log_{10}$) after 10 min of treatment. Based on the extremely rapid inactivation kinetics observed for both the undiluted SD and the SD diluted 1 : 3, it was decided not to perform additional robustness studies.

Precipitation of Cohn fraction III

Precipitation of Cohn Fraction III was studied for BVDV, HIV and PRV and for B19, CPV and EMC. Samples were collected from the suspension after the addition of ethanol, filtrate 1 (≈ 30% of the total filtrate) and filtrate 2 (≈ 70% of the total filtrate), and resuspended paste III (Fig. 4). The rationale for collecting two filtrate fractions was that during filtration, a cake of filter-aid and paste III is gradually built up on the filter surface, which might result in a different efficacy of virus removal early and late during the filtration process. The results show that there is a similar virus reduction obtained for both early and late filtrates, ranging between 3 and 4 $\log_{10}$. The mechanism of virus reduction is removal because insignificant reduction was found in the suspension samples. Moreover, the virus reductions in the resuspended paste III samples were less than 1 $\log_{10}$, confirming that the mechanism is removal.

Virus neutralization

To establish the virus-neutralizing activity of Nanogam®, various dilutions of the final product of Nanogam® were spiked with an intermediate amount (≈ 10^4 $TCID_{50}$/ml) of HAV and BPV and incubated for 30 min at room temperature. Subsequently, the residual infectivity was measured with infectivity tests, and clearance factors were calculated (Fig. 5). Neutralization of HAV and, to a lesser extent, BPV, is clearly demonstrated (i.e. a 3 $\log_{10}$ reduction for the 1 : 27 dilution of Nanogam®).

Discussion

Here we describe the virus safety of a new manufacturing process for a liquid immunoglobulin product, Nanogam®. The Nanogam® manufacturing process includes two effective

Table 4 Factorial design for investigation of the robustness of the 15-nm filtration combined with pepsin treatment at pH 4·4 (pH 4·4/15NF) step for canine parvovirus (CPV)

Run	Source SQ/FRC	Pressure (bar)	Temp. (°C)	Protein (g/l)	pH (–)	Cross-flow (ml/min)	Ionic strength (% NaCl)	Removal (RF)
1	SQ	0·8	35	50	4·4	8	0·3	3·7
2	FRC	0·8	35	50	4·4	8	0·3	4·1
3	SQ	0·6	30	60	4·7	12	0·2	4·4
4	SQ	0·6	40	60	4·1	4	0·45	3·6
5	SQ	1·0	30	40	4·7	4	0·45	4·4
6	SQ	1·0	40	40	4·1	12	0·2	2·9
7	FRC	0·6	30	40	4·1	12	0·45	3·4
8	FRC	0·6	40	40	4·7	4	0·2	3·7
9	FRC	1·0	30	60	4·1	4	0·2	3·6
10	FRC	1·0	40	60	4·7	12	0·45	4·7

The influence of the parameters source of starting material, pressure, temperature, protein concentration, pH, cross-flow and ionic strength were investigated in a robustness study (two-level factorial design). The titres and subsequently reduction factor (RF) values were calculated with the most probable number (MPN) method. In addition the RF values were modelled using linear regression analysis. Runs 1 and 2 are the centre points of the factorial design. The total amount of spiked virus for runs 1–10 was 8·2, 8·1, 8·2, 8·1, 8·3, 8·2, 8·04, 7·9, 7·9 and 8·3 $\log_{10}$, respectively. FRC, Finnish Red Cross Blood Service; SQ, Sanquin.

virus-reducing steps: a combined pH 4·4/15 nm nanofiltration step that is effective for all viruses tested; and an SD treatment step that is effective for all LE viruses tested. Besides these effective steps, we studied the contribution of the precipitation step of Cohn fraction III and of immune neutralization to the total virus-reducing capacity of the manufacturing process.

The combined pH 4·4/15NF step for BVDV, HIV and PRV resulted in reduction factors of > 6·3, > 5·4, and > 6·1 $\log_{10}$, respectively. For CPV, the reduction factors are between 3·5 and 4·5 $\log_{10}$, and for EMC and B19 the reduction factors are > 5·8 and > 6·1 $\log_{10}$, respectively. Based on the virion size of parvovirus (18–26 nm), one may expect complete removal of the virus upon filtration with the 15N filter. However, the 15-nm pore size is not an exact figure, but an average with a particular range (i.e. 15 ± 2 nm), whereas the filter consists of multilayered pore structure of cuprammonium-regenerated cellulose. The effectiveness of virus removal on Planova filters is based on two effects: removal by size exclusion; and removal by depth filtration. As our method for producing the CPV stock generated a considerable amount of non-aggregated virus particles, as demonstrated by filtration with Planova 35N (35 nm; data not shown), this virus spike poses a true worst-case challenge of the filter. To further illustrate the filtration behaviour of parvovirus on a 15N filter, we previously investigated the removal of CPV after single or double serial dead-end filtration with 15N filters. For single filtration we demonstrated a 4 $\log_{10}$ reduction, whereas for double filtration > 6 $\log_{10}$ reduction (F.A.C. van Engelenburg, personal communication) was obtained. The difference in CPV removal between single and double filtration can be explained by additional removal via depth filtration.

A striking observation is the difference obtained in removal for B19 (NAT assay as read-out) vs. the model virus, CPV (infectivity assay as read-out). An explanation for complete removal of B19 can be the presence of neutralizing antibodies in plasma against B19. Omar *et al.* and Troccoli *et al.* [18,23] have shown that antibodies against parvoviruses and enteroviruses in human IgG solutions contribute to enhanced removal of these viruses by nanofiltration. Appreciating that plasma pools, in general, contain large amounts of B19 antibodies, it can be envisaged that the formation of B19–antibody complexes results in the complete removal of B19 after nanofiltration. The results obtained with B19 therefore suggest that the results obtained with CPV underestimate the true virus-reducing capacity of the pH 4·4/15NF step for B19.

In the robustness study of the pH 4·4/15NF step, it was found that pH is the dominant factor. A mechanistic explanation for this result is not quite clear, but subtle matrix effects can play a role. Yokoyama *et al.* [27] found that the addition of 0·3 M glycine to a buffer leads to high reduction of B19 after passing over a Planova 35N filter, whereas in the absence of glycine no reduction was obtained. They also showed that the pH of the glycine buffer had a strong influence on the removal of B19: a shift from pH 4 to pH 6 leads to an additional removal of B19 of ≈ 4 $\log_{10}$. This increased removal may be explained by virus aggregation in the presence of glycine at pH values that are close to the isoelectric points of the B19 capsid proteins, VP1 and VP2 (i.e. pH 6·1 and 6·4, respectively). In the present study, the pH set at the lower limit resulted in a lower RF value and this might be

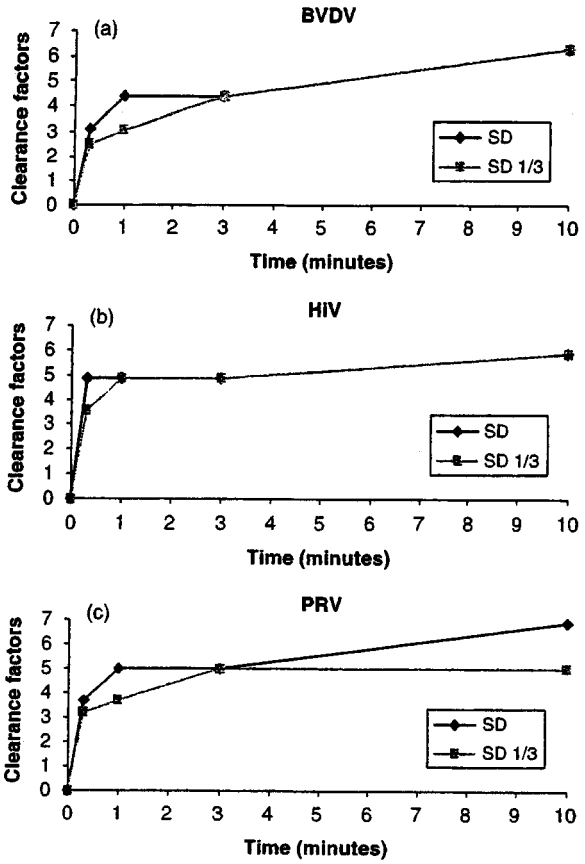

Fig. 3 Inactivation kinetics as result of treatment with the standard solvent–detergent concentration (SD) or the SD concentration diluted 1 : 3 (SD 1/3). (a) Bovine viral diarrhoea virus (BVDV), (b) human immunodeficiency virus (HIV) and (c) pseudorabies virus (PRV). Filled marker, the exact value of the calculated clearance factor (CF) is indicated; open marker, the CF was equal to or higher than the indicated value. The total amount of spiked virus for BVDV was 8·0 $\log_{10}$, for HIV was 7·6 $\log_{10}$, and for PRV was 8·6 $\log_{10}$.

explained in the same way, namely shifting away from the isoelectric point of the virus particle results in less aggregation and therefore in less virus removal.

During tangential flow filtration, a protein layer is potentially built up on the filter. For our filtration process, we have no indications that this is actually the case. However, during the filtration process we observed a decrease in the filtrate flow, from 4 to 1·5 $l/m^2/h$. This decrease can be explained by the notion that the Planova 15N filter consists of layers of filter material encompassing channels with small and large pores. During the filtration of the immunoglobulin solution, the small pores will gradually become blocked with protein, resulting in a decrease of the filtrate flow observed. However, it is the large pores, rather than the small pores, that determine the extent to which viruses will be retained. Therefore, it is not expected that the gradual decrease in filtrate flow will have a major effect on the virus-removing capacity of the filtration step.

For SD treatment, complete inactivation (CF > 6 $\log_{10}$) was found for all LE viruses after only 10 min of treatment. These results are in line with the observations of other investigators [5,8,28,29]. The finding that the SD treatment in the Nanogam® process after 30 s already results in a clearance of more than 5 $\log_{10}$ for PRV, was surprising, in view of the fact that PRV is often the worst-case virus in SD treatment studies. A possible explanation is the relatively low pH (5·0 ± 0·1) of the immunoglobulin solution during incubation and the high sensitivity of PRV to low pH, as can be deduced from the data for pH 4·4 treatment, as previously described. BVDV is relatively more resistant to low pH and therefore was found to be more resistant in the SD treatment in the Nanogam® process than PRV.

Besides the effective virus-reducing steps, the contributing steps (precipitation of Cohn fraction III and immune neutralization) were studied. Precipitation of fraction III resulted,

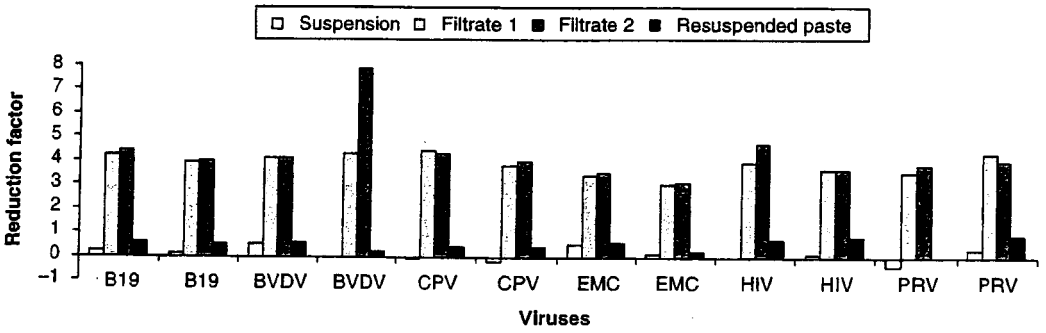

Fig. 4 Precipitation of Cohn fraction III. Cohn fraction III was precipitated and tested for the removal of parvovirus B19 (B19), bovine viral diarrhoea virus (BVDV), canine parvovirus (CPV), encephalomyocarditis virus (EMC), human immunodeficiency virus (HIV) and pseudorabies virus (PRV) (reduction factor values are shown). The results of duplicate runs are shown for the suspension, filtrate 1, filtrate 2 and resuspended paste, respectively. The total amount of spiked virus was 11·4 and 11·0 $\log_{10}$ (B19), 8·4 and 8·6 $\log_{10}$ (BVDV), 9·1 and 9·0 $\log_{10}$ (CPV), 7·7 and 7·3 $\log_{10}$ (EMC), 7·2 and 7·6 $\log_{10}$ (HIV), and 8·3 and 8·6 $\log_{10}$ (PRV) for runs A and B, respectively.

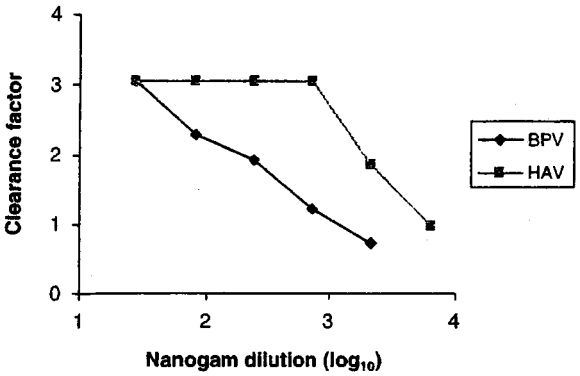

Fig. 5 Neutralization capacity of Nanogam®. Various dilutions of the final product of Nanogam® (source Dutch plasma) were spiked with intermediate amounts [≈ 10^4 tissue culture infectious dose 50% ($TCID_{50}$)/ml] of bovine parvovirus (BPV) and hepatitis A virus (HAV) and incubated for 30 min. Depicted are the clearance factors for the 1 : 27-, 1 : 81-, 1 : 243-, 1 : 729-, 1 : 2187- and 1 : 6561-diluted final product of Nanogam®.

for the relevant filtrates, in reduction factors of 3 - 4 log_{10} for all viruses tested. In a study of Bos *et al.* [3] the effect of removing the paste via centrifugation vs. filtration for the precipitation of fraction III was investigated. In this experimental set-up, the centrifugation step was performed in the absence of filter aid, whereas the filtration was performed in the presence of filter aid (J. Over, personal communication). For PRV and SV40, Bos *et al.* showed that the results obtained for the suspension, supernatant and precipitate fractions were similar. Therefore, these results suggest that the partitioning is caused by ethanol precipitation per se and that the contribution of the separation method (i.e. addition of filter aid and filtration) is likely to be limited. Overall, our data indicate that precipitation of fraction III can be considered as a reproducible contributing virus-removal step [30].

The presence of virus-neutralizing antibodies contributes to the safety of a product. We showed significant neutralizing activity, in the final product, for BPV and HAV. For BPV it was found that a dilution of 1 : 27 of the final product still resulted in neutralization of more than 3 log_{10}. The neutralization found for BPV can be an underestimation of the total capacity to neutralize B19, as cross-neutralization of BPV by B19 antibodies might be less effective than neutralization of B19. For HAV, the neutralizing effect was more pronounced; even a dilution of ≈ 1 : 729 resulted in neutralization of more than 3 log_{10}. However, when evaluating the total virus-reducing capacity of the manufacturing process, it is, in general, difficult to assess the contribution of immune neutralization in the final product. In contrast to inactivating, removing or partitioning steps, where there is a direct effect on the virus, either by physical or chemical disruption of the virus particle, or by physical removal of the virus from the product, in the event of immune neutralization, the potentially infectious virus remains present as a complex with antibodies. In theory, such complexes might dissociate and the virus may regain its infectivity [31].

Neutralizing antibodies are present in both plasma pools and final products, and contribute to the viral safety of Nanogam®. To obtain insight into the levels of neutralizing antibodies in the final product, a series of batches derived from Finnish and Dutch plasma have been tested for antibodies. In Finnish blood donors, the anti-B19 concentrations were 800 and 3200 (B19 ELISA units) and for anti-HAV were 1·1 and 11·7 IU/ml in plasma and final product, respectively. In Dutch blood donors, the anti-HAV concentrations were 4 and 30 IU/ml in plasma and final product, respectively - no data were available for the anti-B19 concentrations (A.H.L. Koenderman, personal communication). Based on the epidemiology of B19, and the age distribution of blood donors, it is unlikely that the anti-B19 levels will change in the next decade. B19 has a stable epidemiology worldwide; it can be expected that the level of neutralizing antibodies in the final product will remain constant. In the age group of the majority of blood donors (i.e. between 21 and 60 years), 57 - 84% have seroconverted for B19 [32]. For BPV, we demonstrated immune neutralization of more than 4 log_{10}. Bearing in mind some minor fluctuations in anti-B19 levels, we propose inclusion of a safety margin for B19 of 1 log_{10}, resulting in a contribution of neutralization in the final product of at least 3 log_{10}. This estimation is supported by other investigators [33].

For HAV it has been shown that 50% of the Dutch blood donors have seroconverted for HAV infection by the age of 40 years [34]. Based on the epidemiology of HAV and the age distribution and socioeconomic status of blood donors, it is likely that the anti-HAV levels in plasma pools will slowly decrease in the next decade, [35,36]. Despite this expected decrease in seroprevelance the current levels of anti-HAV antibodies are very high in Dutch plasma (4 IU/ml). Appreciating a slowly declining level of anti-HAV antibodies, we are reluctant to use the demonstrated high reduction value for the contribution of immune neutralization i.e. more than 6 log_{10} for HAV and therefore we propose to include an additional safety margin for HAV of 3 log_{10}, resulting in contribution of neutralization of at least 3 log_{10}.

To obtain insight into the robustness of immune neutralization, but also of the contribution of the presence of virus-specific antibodies during nanofiltration, further research will be necessary. This research will assist in setting limits for minimal levels of virus-specific antibodies for assuring the demonstrated viral reductions for B19 and HAV of the respective steps.

For the Nanogam® production process, three different steps were studied: a precipitation step of the Cohn fractionation process (partitioning of viruses in ethanol-containing protein

Table 5 Summary of virus-reduction values obtained for Nanogam®

Step	B19	BVDV	CPV (BPV)	EMC (HAV)	HIV	PRV
Precipitation fraction III	3·9	4·1	3·8	3·0	3·6	3·5
pH 4·4/15NF treatment	> 6·1	> 6·3	> 3·4	> 5·8	> 5·4	> 6·1
SD treatment	NA	> 6·3	NA	NA	> 5·9	> 6·9
Neutralization	NA	NA	(3)	(3)	NA	NA
Total reduction	> 10	> 16·7	> 10·2	> 11·8	> 14·9	> 16·5

B19, parvovirus B19; BPV, bovine parvovirus; BVDV, bovine viral diarrhoea virus; CPV, canine parvovirus; EMC, encephalomyocarditis virus; HAV, hepatitis A virus; HIV, human immunodeficiency virus; PRV, pseudorabies virus.
pH 4·4/15NF, 15-nm filtration step combined with pepsin treatment at pH 4·4; SD, solvent–detergent.

solutions at a particular pH and temperature); a nanofiltration step (virus removal by size exclusion and depth filtration); and an SD-treatment step (viral inactivation of LE viruses by disruption of the viral membrane). As the mechanisms of viral reduction are different, the process steps tested show a reproducible reduction for a broad series of viruses, and because the process steps are robust, it is acceptable to sum up individual RF values to estimate the overall virus-reducing capacity of the production process. Summing up the RF values obtained leads to > 15 $\log_{10}$ reduction for the LE viruses and > 10 $\log_{10}$ reduction for the NLE viruses (Table 5). Therefore, we conclude that the Nanogam® product meets the latest requirements for virus inactivation/removal and can be considered to be a viral-safe product.

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別紙 3-5

医薬品
医薬部外品　研究報告　調査報告書
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識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2006 年 1 月 30 日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称			研究報告の公表状況	A modified caprylic acid method for manufacturing immunoglobulin G from human plasma with high yield and efficient virus clearance J. Parkkinen, A. Rahola, L. von Bonsdorff, H. Tölö & E. Törmä Vox Sanguinis 2006; 90; 97- 104	公表国 米国	
販売名（企業名）						

研究報告の概要

静注用免疫グロブリン製剤の開発において高収率で IgG が得られる血漿分画法が求められている。本稿では、不活化工程に対して物理化学的に耐性を示すウイルスを効率的に除去しつつ高い収率で IgG を得られる新規の製造法が提示されている。
これまでに、分画 II+IIII から開始してカプリル酸塩沈降法とクロマトグラフィーを組み合わせた精製工程が発表されているが、カプリル酸沈降法は有効なウイルス不活化工程と精製工程を併せ持つ工程として提示されている。
本研究では、人血漿から高収率で IgG を精製するための変法カプリル酸法について検討した。濾過条件を最適化し、重合化蛋白を除去することで生成物はウイルス濾過フィルターを容易に通ることができる。この方法により、パルボウイルス B19 などのエンベロプをもたない小さなウイルスを非常に高い確率で除去することが可能になる。
本方法では、分画 II+III にカプリル酸塩を添加し、その後 PEG 処理を行った。さらに陰イオン交換クロマトグラフィーを行うことで純度の高い IgG が高収率で得られた。カプリル酸処理によるウイルス除去能は 10^{4} であり、全工程におけるウイルス除去能は $10^{13.5}$ と非常に高かった。この製造法が実用化されれば、分画 II+III から安定かつ重合体を含まない IgG が高収率で得られる（すなわちウイルス除去フィルターにより濾過ができる）ことが示された。

使用上の注意記載状況・その他参考事項等

BYL-2005-0204

報告企業の意見	今後の対応
弊社の静注用免疫グロブリン製剤の製造工程においてカプリル酸および PEG 処理は行われていないが、本剤のウイルス不活化工程（分画、透析・限外濾過、S/D 処理、低 pH インキュベーション）によるパルボウイルス B19 のモデルウイルスの除去率は $10^{9.2}$ 以上と非常に高い。しかしながら現時点でもなお感染の可能性を完全には否定できない。本稿において示されたパルボウイルス B19 の不活化工程の実用化については引き続き注視して情報を収集する。	現時点で新たな安全対策上の措置を講じる必要は無いと考える。引き続きパルボウイルス B19 の除去および検出技術に関する関連情報の収集に努める。

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ORIGINAL PAPER

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A modified caprylic acid method for manufacturing immunoglobulin G from human plasma with high yield and efficient virus clearance

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Vox Sanguinis

Background and Objectives The increasing demand for intravenous immunoglobulin (IVIG) necessitates the development of improved plasma fractionation methods, providing higher immunoglobulin G (IgG) recovery. Here, we describe a new IVIG production process resulting in a high yield of IgG and effective reduction of physico-chemically resistant viruses.

Materials and Methods IgG was purified from Cohn fraction II+III by caprylic acid treatment, polyethylene glycol precipitation, anion-exchange chromatography, nanofiltration and ultrafiltration. Stability of the purified IgG was studied in different formulations. Virus reduction was studied with two viruses: bovine viral diarrhoea virus, assessed by an infectivity assay; and human parvovirus B19, assessed by polymerase chain reaction.

Results The combination of caprylic acid treatment with polyethylene glycol precipitation and a single anion-exchange chromatography yielded polymer-free, pure IgG. The purified IgG could be filtered through a small pore-size virus filter (Millipore V-NFP) with high throughput and excellent yield. The formulated product was stable as a 100 g/l IgG solution. Bovine viral diarrhoea virus was effectively inactivated by the caprylic acid treatment, and parvovirus B19 was effectively removed in the polyethylene glycol precipitation and nanofiltration stages, the total reduction of parvovirus being $\approx 14 \log_{10}$.

Conclusions The new process gives pure and stable IgG solution with an average yield of 4·8 g of IgG per kg of recovered plasma and has a very high capacity to remove even physico-chemically resistant viruses.

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Key words: caprylic acid, IgG, intravenous immunoglobulin, nanofiltration, parvovirus, polyethylene glycol.

Introduction

Intravenous immunoglobulins (IVIGs) are concentrated formulations of human immunoglobulin G (IgG) that are prepared by the industrial fractionation of large pools of individual plasma donations. IVIG is widely used for the treatment of patients with primary and secondary immune deficiencies, as well as for the treatment of various immune-mediated disorders, such as thrombocytopenic purpura and polyneuropathies [1–3].

IgG has traditionally been separated in large scale from human plasma by the cold ethanol fractionation method developed in the 1940s [4,5] and its subsequent modifications. The early IgG preparations could only be administered intramuscularly or subcutaneously because of adverse effects associated with their intravenous infusion [6]. These adverse effects were mainly caused by immunoglobulin aggregates inducing complement activation and occasionally by

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