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# Characterisation of a chromatographically produced anti-D immunoglobulin product

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**Abstract**

A chromatographic fractionation method has been developed for the production of a liquid-stable anti-D immunoglobulin product for intravenous and intramuscular use. An immunoglobulin fraction, highly enriched with anti-D immunoglobulins, was isolated by cation-exchange column chromatography and further polished, first by anion-exchange chromatography, followed by an aluminium hydroxide gel treatment. The process includes two specific steps for virus inactivation and removal, namely S/D treatment and nanofiltration. The overall anti-D process yield is about 56%. The final product is stabilised with human albumin and glycine and placed in ready-to-use syringes. The anti-D product was shown to be stable in liquid state for at least 30 months at 4°C. © 1997 Elsevier Science B.V.

*Keywords:* Nanofiltration; Anti-D immunoglobulin; Hyperimmunoglobulin

## 1. Introduction

Before the introduction of anti-D immunoglobulin (anti-D Ig) products for the prevention of Rhesus (Rh) immunisation in the late 1960s, haemolytic disease of the new-born (HDN) due to Rh blood group incompatibility was a serious cause of prenatal mortality [1]. Ever since, Rh prophylaxis by the injection of anti-D Ig to Rh D-negative mothers who gave birth to a Rh D-positive child has become routine in many countries [2,3]. The risk for HDN could further be reduced by routine antepartal administration of a dose of anti-D Ig at 28–30 weeks gestation [2,4]. Anti-D Ig is also used for the suppression of Rh immunisation in nonsensitized Rh D-negative women after abortions, amniocentesis, chorionic villus sampling, ruptured tubal pregnancy or abdominal or transplacental haemorrhage, as well as in cases of mistransfusion of Rh D-positive red blood cells (rbc) or transfusion of blood components containing Rh D-positive rbc to Rh D-negative recipients. Finally, anti-D Ig is also used for the treatment of idiopathic thrombocytopenic purpura (ITP) [5], although efficacy here remains controversial [6,7].

Due to an increasing need for anti-D Ig and because of the successful prevention of Rh immunisation, decreasing numbers of naturally immunised women who could be potential anti-D plasma donors, the supply of anti-D Ig products has become a wide problem. Therefore Rh-negative female and male volunteers have to be recruited for immunisa-

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tion with Rh D-positive rbc. Comprehensive safeguards governing immunisation and boosting, as well as collection of anti-D plasma including viral and antibody testing of donors and rbc, quarantine of frozen rbc, recipient testing and testing of the hyperimmune plasma have to be performed. This makes anti-D hyperimmune plasma both rare and costly. Clinical use of monoclonal or recombinant anti-D instead of classical anti-D Ig products would eliminate the need to recruit and immunise volunteer donors. However, until a monoclonal anti-D becomes available and is shown to have the same degree of efficacy as plasma-derived polyvalent anti-D products, economic and efficient methods for the preparation of safe and efficacious anti-D Ig products are needed. In the past, anti-D Igs were produced by cold ethanol fractionation [8,9] or by column chromatography on anion exchangers [10–13]. Most anti-D products are still produced by ethanol fractionation. Only few products are obtained exclusively by chromatography, especially after evidence of transmission of hepatitis C virus to patients when the manufacturing process did not include efficient viral inactivation and/or removal steps [14,15]. According to our experience, ethanol fractionation of anti-D Ig results in extremely low yields (below 20%), whereas yields of 90% were reported for anti-D Ig purified by a single anion-exchange chromatography [13].

Based on these considerations we developed a multistep chromatographic fractionation method for the production of a safe and liquid-stable anti-D Ig preparation for intravenous and intramuscular use. The process includes two specific steps for virus inactivation and removal, namely S/D treatment [16,17] and nanofiltration [18]. Source plasma containing anti-D immunoglobulins was obtained by plasmapheresis of Rh-immunised volunteers.

Extensive process and virus validation studies, as well as stability studies were performed. Data on process validation, virus validation and product stability are presented.

## 2. Experimental

### 2.1. Purification of anti-D IgG

The starting material was plasma from Rh D-negative women or men who were sensitised against Rh factor D with carefully selected Rh D-positive rbc. The plasma obtained through plasmapheresis was individually frozen and thawed at 0–4°C prior to fractionation and pooled to 25 l. The plasma pool was centrifuged at 9400 *g* with a continuous flow of 1 l/min in a Cepa centrifuge (Cepa, Lahn, Germany) to remove the cryoglobulins. Enveloped viruses (e.g. such as HIV, Hepatitis B and Hepatitis C) that potentially could have been present were then inactivated by S/D treatment of the cryoglobulin depleted plasma pool with 1% Triton X-100 (Rohm and Haas, Frankfurt, Germany) and 1% tri-*n*-butylphosphate (TnBP, Merck, Darmstadt, Germany) for 4–4.5 h at 30°C [16,17]. S/D-treated plasma was then fractionated on a weak cation exchanger (MacroPrep 50 CM, BioRad, Hercules, CA, USA). Plasma was first diluted with 10 mmol/l sodium phosphate to the conductivity of the equilibration buffer (50 mmol/l sodium phosphate buffer, pH 5.5, 3.2 mS/cm), the pH adjusted to 5.5, filtered through a 1.2-μm Sealkleen 7002 NNP filter (Pall, Dreieich, Germany) and then immediately chromatographed on MacroPrep 50 CM in a glass column (30×7.5 cm I.D.) at a flow-rate of 150 cm/h. The column was subsequently washed with 40 column-volumes of 25 mmol/l sodium phosphate buffer, pH 7.0, and the anti-D IgG-enriched fraction was eluted with 1 column-volume of 25 mmol/l sodium phosphate buffer, pH 7.5, containing 0.2 mol/l sodium chloride. Further purification was performed by adsorption of impurities with a weak anion exchanger (DEAE-Sephadex, Pharmacia, Uppsala, Sweden) with 2 g per l, followed by an aluminium hydroxide gel (Alhydrogel, Superfos, Vedbaek, Denmark) with 0.2 g $Al(OH)_3$ per g protein in batches. To achieve further concentration, the purified anti-D IgG fraction was again bound to MacroPrep 50 CM in a glass column (12×10 cm I.D.) with a flow-rate of 230 cm/h, the gel having been previously equilibrated with 50 mmol/l sodium phosphate buffer, pH 5.5. The anti-D containing IgG fraction was eluted with 25 mmol/l sodium phosphate buffer, 0.2 mol/l sodium chloride, pH 5.5. MacroPrep 50 CM columns can be reused at least 20 times without deterioration. Columns were cleaned in place with 2 column volumes of 1 mol/l NaCl and 5 column-volumes of 1 mol/l NaOH, finally rinsed with 4 column volumes of water and stored in 20% ethanol. DEAE-Sephadex and Alhydrogel were both discarded after single use.

### 2.2. Nanofiltration

Nanofiltration for virus removal with Planova 15N was performed according to the manufacturer's recommendations (Asahi Chemical Industry, Tokyo, Japan). The filter technology is based on hollow fibre membranes made from cuprammonium regenerated cellulose. The concentrated anti-D IgG fraction produced as described above was prefiltered at a protein concentration of 8–10 g/l through a 0.1-μm Sealkleen 7001 NTP filter (Pall) and immediately nanofiltered through a Planova 15N filter (15 nm mean pore size) with a maximum volume of 20 $l/m^2$. The nanofiltration was performed in dead-end mode at room temperature at a maximum pressure of $10^5$ Pa. Total protein, total IgG and anti-D antibodies were analysed, and yields were calculated. Protein composition (SDS-PAGE) and size distribution (HPLC) before and after nanofiltration were compared. SDS-PAGE was performed as described [19]; HPLC was performed on a TSK G3000SW column 60×7.5 mm I.D. (TosoHaas, Stuttgart, Germany) with a 40 mmol/l sodium phosphate buffer, pH 7.0, at a flow-rate of 0.5 ml/min on a HP1050 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany).

### 2.3. Formulation

The bulk solution, as described above, was diluted to a final concentration of 100 μg/ml anti-D IgG and stabilisers were added (final concentrations being 1% human albumin (Albumin SRK, ZLB, Bern, Switzerland) and 275 mmol/l glycine (Merck), respectively). The pH was adjusted to 5.2; after sterile filtration the solution was dispensed into 2-ml ready-to-use syringes.

### 2.4. Virus validation

Virus validation studies were performed according to established European Community (EC) guidelines [20–22]. The studies were performed at our institution, or in collaboration with CLB (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) or Analysis Biomedizinische GmbH (Frankfurt, Germany). Six individual steps employed in the production of the anti-D product, which involved cation- and anion-exchange chromatography, treatment with aluminium hydroxide gel, S/D treatment as well as nanofiltration, were validated for their effectiveness in inactivating and/or removing viruses. Viruses used in these validation studies were chosen according to the recommendations of the European Committee of Proprietary Medicinal Products (CPMP) [21]: human immunodeficiency virus (HIV), as a model for HIV 1 and HIV 2; bovine viral diarrhoea virus (BVDV), as a model virus for hepatitis C virus; pseudorabies virus (PRV), as a model for large enveloped DNA viruses; and canine parvovirus (CPV), as a model for human parvovirus B19.

### 2.5. Analytical techniques

Anti-D potency was measured by the autoanalyser-haemagglutination technique described in the European Pharmacopoeia (Ph. Eur.) [23] using a WHO reference anti-D as standard. IgG, IgG-subclasses and IgM were determined by laser nephelometry (Nephelometer BNA, Behringwerke, Marburg, Germany). IgA was measured by radial immunodiffusion using NANORID (The Binding Site, Birmingham, UK). Relative contents of plasma proteins were determined by cellulose acetate membrane electrophoresis with the Celloplaque kit (Sebia, Issy-les-Moulineaux, France). Molecular size distribution (monomers, dimers, aggregates) was determined by HPLC as described above (see Section 2.2). Anticomplementary activity (ACA), osmolality and prekallikrein activator (PKA) were assayed according to the methods described in Ph. Eur. [24]. Activities of serine proteases were measured with the chromogenic substrate S-2288 (Chromogenix, Mölndal, Sweden). Antibody-dependent cell-mediated cytotoxicity tests (ADCC) were performed as described [25]. Briefly, group 0 Rhesus-positive rbc (=target cells) were incubated with 1% papain (Fluka, Buchs, Switzerland) in sterile saline for 4 min at room temperature, and subsequently labelled with $^{51}Cr$ (NEN, Boston, MA, USA). The radiolabelled rbc were preincubated with anti-D products in serial dilutions for 1 h at 37°C. Then, monocyte-depleted mononuclear cells (=effector cells) were added to the rbc and the cell suspension was incubated for 18 h at 37°C. $^{51}Cr$ release was measured in a 1215 Rackbeta counter

(Wallac Oy, Turku, Finland) and specific lysis was calculated.

### 2.6. Stability testing

Anti-D potency, total protein, pH, ACA, PKA and osmolality were assayed at the beginning of the study and after 6, 12, 18 and 24 months of storage at 4 and 26°C, and additionally, after 30 months of storage at 4°C. Anti-D potency was also tested in a 'stress test', after an additional incubation at 37°C for 4 weeks at each time point.

## 3. Results and discussion

### 3.1. Purification process

The anti-D immunoglobulin is purified by a combination of antibody adsorption and desorption on different chromatographic resins. The isolation method includes two specific steps for virus inactivation and removal, namely S/D treatment and nanofiltration. Anti-D and total protein recoveries during the production process are summarised in Table 1.

The main purification of anti-D IgG was performed by cation-exchange chromatography on a MacroPrep 50 CM column. Albumin, α- and β-globulins as well as a substantial part of γ-globulins, comprising more than 90% of IgG and about 99.7% of IgA were washed out from the MacroPrep 50 CM column, whereas most of the specific anti-D IgG antibodies were retained by the column. The anti-D IgG-enriched fraction was then eluted with a buffer of increased ionic strength. A typical elution pattern is shown in Fig. 1.

In contrast to previously described chromatographic methods [10–13], where anion exchangers were used to isolate the complete γ-globulin fraction, the method described herein, makes use of a cation-exchange chromatography as a principle isolation step that allows the isolation of anti-D IgG in a significantly higher purity. The final product, containing about 1% of the original total protein and $<10\%$ of the total IgG, had a significantly increased specific anti-D antibody activity. The specific activity was directly depending on the plasma pool anti-D concentration and varied from 2.8 to 7.3% ($n=7$). The IgG subclass distribution was significantly altered by this step: $IgG_1$ and $IgG_3$ subclasses were enriched from approximately 67 to 84% and from 7.5 to 9%, respectively, whereas $IgG_2$ was depleted from 22 to 7%, and $IgG_4$ from 4% to trace. Anti-D activity is mainly associated with subclasses $IgG_1$ and $IgG_3$; therefore, the enrichment of these subclasses is a reason for the increased specific activity of anti-D IgG. The same chromatographic step reduced

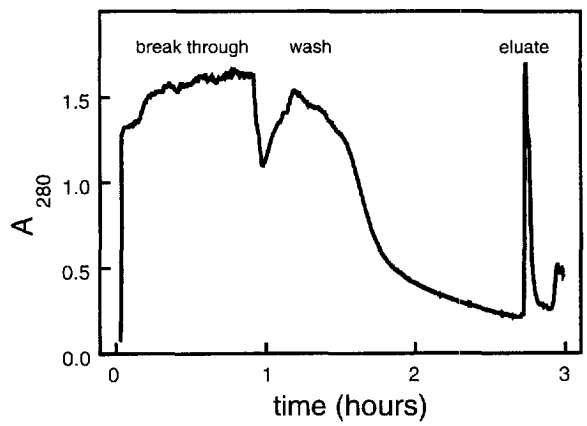


Fig. 1. Chromatographic pattern of plasma protein fractionation by cation-exchange column chromatography on MacroPrep 50 CM. The sharp elution peak reflects an IgG fraction with selectively enriched anti-D IgG.

Table 1
Anti-D and protein recovery during production process

| Sample | Anti-D recovery (mean±S.D., $n=7$) (%) | Protein recovery (mean±S.D., $n=7$) (%) |
|---|---|---|
| Cryoprecipitate-depleted plasma | 100 | 100 |
| Post S/D-treatment | 98.4±2.7 | 102.1±5.9 |
| Post cation-exchange chromatography | 71.1±4.5 | 1.5±0.1 |
| Post DEAE-Sephadex treatment | 64.0±5.2 | 1.1±0.1 |
| Post Alhydrogel treatment | 55.7±3.5 | 1.0±0.1 |
| Post concentration | 55.9±4.5 | 0.9±0.1 |

Table 2
Composition of bulk solution

| Analyses | Mean±S.D. ($n=7$) (%) |
|---|---|
| Gammaglobulins | 98.3±0.9 |
| IgG 1 | 83.8±1.7 |
| IgG 2 | 7.1±2.4 |
| IgG 3 | 8.7±1.1 |
| IgG 4 | traces |
| Monomers + dimers | 100±0.1 |
| Aggregates | 0.0±0.1 |

the amount of solvent and detergent used for virus inactivation to <1 and <5 μg/ml for Triton X-100 and TnBP, respectively.

Further purification of anti-D IgG was achieved by treatment with a weak basic ion-exchange gel (DEAE-Sephadex), followed by an aluminium hydroxide gel (Alhydrogel). The remaining IgA was specifically reduced by >95%, to <5 μg/ml ($n=6$) by the DEAE-Sephadex treatment, whereas serine proteases were reduced by >96%, to <5 nkat/l ($n=7$) by the treatment with Alhydrogel. Thus the final anti-D product contains very low concentrations of IgA (<0.25 μg/ml). Such low amounts of IgA in immunoglobulin products were reported to be well tolerated even by IgA hypersensitive IgA-deficient patients [26]. An improved tolerability is therefore expected for our anti-D product.

Subsequently, the purified anti-D IgG fraction was concentrated by a second cation-exchange chromatography on MacroPrep 50 CM gel and eluted in a small volume with a buffer of increased ionic strength.

Purity and composition of this bulk solution are summarised in Table 2. Table 3 shows the correlation between the anti-D concentration in the plasma pool and the specific activities of the anti-D IgG that were obtained by this procedure. Taking into account that plasma pools have an IgG concentration of approximately 10 mg/ml, the increase in specific activity is about 10-fold. This also holds true if, for process validation, very low titre plasma pools (<1 μg/ml anti-D) were fractionated (data not shown). Yield and purity of laboratory, pilot and process scale products were very consistent and no problems arose during the scaling up procedure. The overall anti-D yield of seven production lots was 56±5%. According to our experience and reports from others, significantly lower yields were obtained for anti-D Ig produced by ethanol fractionation. No data on other chromatographically produced commercial anti-D products are available. Characteristics of the final product are listed in Table 4.

Table 4
Composition of final product

| Volume | 2 ml |
|---|---|
| Anti-D IgG | 200 μg |
| Albumin | 20 mg |
| Protein content | <30 mg |
| Glycine | 41.2 mg |
| pH | 5.2 |

The final product contains 1.4–3.9 mg/ml of IgG, <5 μg/ml of IgM and <0.25 μg/ml of IgA ($n=7$). The variation in IgG content is due to the variation of the anti-D titre of the source plasma.

### 3.2. Functionality

Potency and functionality of the described anti-D product were demonstrated in vitro, generally by the autoanalyser haemagglutination assay that was used

Table 3
Specific activity

| Lot no. | Content of anti-D IgG in plasma pool (μg/ml) | Specific activity in final product (%) |
|---|---|---|
| 1 | 25.8 | 2.8 |
| 2 | 60.9 | 5.5 |
| 3 | 55.9 | 4.5 |
| 4 | 54.8 | 5.6 |
| 5 | 64.7 | 5.2 |
| 6 | 77.3 | 7.3 |
| 7 | 37.7 | 3.2 |

Specific activity is expressed as % of specific anti-D IgG molecules per total IgG.

for the quantification of anti-D (in-process, on the final product and during stability studies), and additionally, by comparison with Immunglobulin Anti-D SRK (the predecessor product of ZLB), in an ADCC assays (Fig. 2).

### 3.3. Stability

Long-term stability of the anti-D product was investigated with material produced in both pilot and production scale. The potency, defined as anti-D antibody content, did not change after 30 months of storage at 4°C (Fig. 3). A continuous decrease in anti-D antibody activity was observed at a storage temperature of 26°C resulting in a 10% loss of activity within 6 months (Fig. 3), although no degradation of the IgG was apparent in SDS-PAGE (data not shown). All other parameters measured (total protein, pH, ACA, PKA, osmolality) did not change during the study at either temperature. Thus, the anti-D product is stable for at least 30 months at 4°C but only for a period of about 6 months at 26°C. The stability of various liquid anti-D preparations from different European manufacturers has been previously described [27,28]. The loss of activity at 4°C varied from about 1% to more than 10% per year. This variation was attributed to residual proteolytic activities, as well as the problem of the error of the anti-D quantification assay. The described anti-D product shows very low serine protease (including plasmin) activity. Plasminogen and PKA were below detection limits. These properties, i.e. high purity and low proteolytic activities, combined with an optimised formulation, contribute to the excellent stability of the liquid product at 4°C. The accelerated degradation test performed after heating the final product to 37°C for 4 weeks (stress test according to Ph. Eur.) never exceeded a drop of 20% of the initial anti-D value. Thus all results were in compliance with the requirements of the Ph. Eur. monographs on Human Anti-D Immunoglobulin and Human Immunoglobulin for Intravenous Use [23,29].

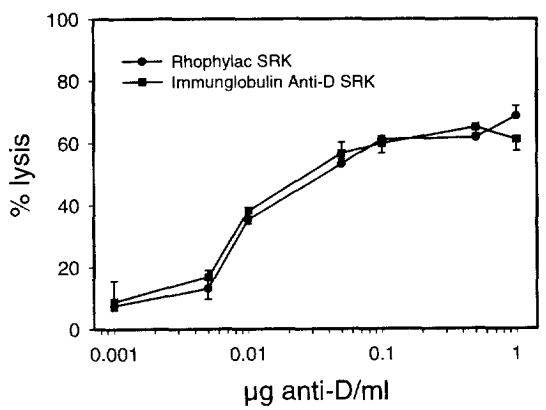


Fig. 2. The new anti-D product and Immunglobulin Anti-D SRK showed the same efficacy when compared in an ADCC assay. Results are expressed as % lysis of three independent experiments (mean±S.D.).

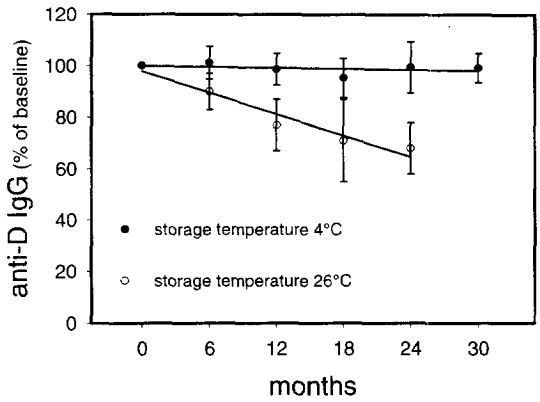


Fig. 3. Anti-D stability data (mean±S.D. with regression lines) of different lots, produced in pilot or production scale. Samples were stored at 4°C for up to 30 months ($n=7$) and at 26°C for up to 12 months ($n=5$) or up to 24 moths ($n=3$), respectively, in the final glass container (ready-to-use syringe). Data are expressed as % of baseline.

### 3.4. Safety

Six different steps of the production process, which involved cation-exchange columns I and II, treatment with DEAE-Sephadex, treatment with Alhydrogel followed by filtration, S/D treatment as well as nanofiltration were validated for their potency of virus inactivation and/or removal. Duplicate experiments were performed with in-process fractions from two individual production lots. The minimal cumulative reduction factors for the individual viruses are listed in Table 5.

The viral elimination capacity of single chromatographic purification steps was reported to be in-

Table 5
Cumulative virus reduction factors ($\log_{10}$)

| | Virus | | | |
|---|---|---|---|---|
| | HIV | BVDV | PRV | CPV |
| Genome: | RNA | RNA | DNA | DNA |
| Envelope: | yes | yes | yes | no |
| Size (nm): | 80–100 | 40–70 | 120–200 | 18–24 |
| S/D treatment | ≥6.0 | ≥5.4 | ≥5.6 | n.d. |
| Cation-exchange column I | –[a] | 1.6 | –[a] | 2.5 |
| DEAE treatment | 1.4 | –[a] | ≥2.5 | –[a] |
| Alhydrogel treatment | 3.1 | –[a] | 1.4 | –[a] |
| Cation-exchange column II | –[a] | –[a] | –[a] | –[a] |
| Nanofiltration | ≥6.3 | ≥5.5 | ≥5.6 | 3.5 |
| Total | ≥16.8 | ≥12.5 | ≥15.1 | 6.0 |

n.d., not determined.
[a]No significant reduction.

sufficient [30]. We also found some contribution to the overall virus reduction by distinct chromatographic steps with certain viruses, but these steps cannot replace specific virus inactivation and/or removal treatments. Efficacy of S/D treatment for the inactivation of enveloped viruses, as well as the safety of S/D-treated blood products are well established and widely accepted [31,32]. S/D treatment of pool plasma did not significantly affect anti-D functionality and was therefore the method of choice for virus inactivation. In the present study, this process was further investigated in order to demonstrate the kinetics of inactivation. All three enveloped viruses tested (HIV, BVDV, PRV) were inactivated to undetectable levels within 2 min.

Nanofilters for removal of viruses in the purification process of plasma products only recently became available. Because nanofiltration removes viruses according to their size, it is applicable to all types of viruses above the cut-off of the filters used. The efficacy of nanofiltration as a method for improving the safety of plasma products has to be carefully considered [33,34]; in particular, the pore size has to be chosen as small as possible so that viruses are removed effectively, but not so small that the proteins of interest are retained by the filter. So far, there are only a few nanofiltered plasma products described in the literature [35–38]. Nanofiltration of bulk solution was introduced into the purification process to increase the viral safety of the anti-D product. A 15-nm pore size filter was chosen since this should remove even very small, non-enveloped viruses. No differences in protein composition (SDS-PAGE) and size distribution (HPLC) were detectable before and after nanofiltration. No significant loss of proteins was observed. Recoveries of total protein, total IgG and anti-D antibodies in the nanofiltrate were 94.3±2.5, 99.2±4.2 and 95.5±1.9%, respectively ($n=6$).

The two specific steps for virus inactivation and removal, namely S/D treatment and nanofiltration, contribute to the high level of virus safety. The cumulative virus reduction factors that were obtained in the validation studies (Table 5) are in compliance with current regulatory requirements [20–22]. Additional safety is obtained by extensive testing of each single donation for absence of HBsAg, antibodies against HIV 1+2 and HCV, as well as absence of increased ALAT (alanine aminotransferase) activity. In addition, plasma pools are screened by PCR for HAV and parvovirus B19. The anti-D product contains substantial amounts of antibodies against hepatitis A virus and human parvovirus B19. Such antibodies may greatly contribute to the overall viral safety by neutralising these viruses if present in the plasma pool.

Tolerability, viral safety, as well as the efficacy to prevent Rh immunisation have been demonstrated in two clinical studies. Pharmacodynamics, pharmacokinetics and efficacy were investigated in a phase-I, single-centre, open-label, single-dose study with Rh-negative male volunteers challenged with

Rh-positive red blood cells. A phase-II/III multicentre, open-label, single-dose study demonstrated the tolerability and efficacy to prevent Rh immunisation of Rh-negative postpartum mothers at risk of developing Rh antibodies.

## 4. Conclusions

The anti-D product described in this report is highly pure, liquid-stable, safe and can be administered either by the i.v. or the i.m. route. Safety, efficacy and tolerability have been demonstrated in two clinical studies. No adverse events related to the use of the anti-D product have been reported within the first year of its routine clinical use. The excellent yield that is obtained by the chromatographic purification process saves rare and costly anti-D plasma and may help to overcome the wide shortage of anti-D products.

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