



Comparative removal of solvent and detergent viral inactivating agents from human intravenous immunoglobulin G preparations using SDR HyperD and C18 sorbents

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ABSTRACT

The capacity of hydrophobic octadecyl (C18) and SDR HyperD materials to remove the combination of 1% (v/v) solvent (tri-*n*-butyl phosphate, TnBP) with 1% (v/v) nonionic detergents (Triton X 100 and Triton X 45) used for viral inactivation of plasma derived polyvalent intravenous immunoglobulin G (IVIG) preparation has been evaluated. Efficient removal of TnBP (<10 ppm in IVIG preparation) was found at ratios of 0.5 g of C18/7 ml of IVIG and 0.22 g of dry SDR HyperD/7 ml of IVIG. Binding capacities of TnBP were greater than 140 mg/g of C18 and greater than 318 mg/g of dry SDR HyperD. Complete removal of Triton X 45 (<2 ppm) was obtained at ratios of 1 g of C18/7 ml of IVIG and 0.44 g of dry SDR HyperD/7 ml of IVIG or above, corresponding to binding capacities in excess of 70 mg/g of C18 and in excess of 159 mg/g of dry SDR HyperD. Residual Triton X 100 was less than 30 ppm at a ratio of 4 g/14 ml of immunoglobulin G (IgG) for the C18 sorbent. Triton X 100 was less than 10 ppm when using SDR HyperD at a ratio of 0.66 g/7 ml of IgG, corresponding to a binding capacity of approximately 106 mg of Triton X 100/g of dry SDR HyperD. Good recoveries of IVIG were achieved in the effluent from both sorbents.

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Polyvalent intravenous immunoglobulins G (IVIGs)¹ are currently the driving protein fraction behind the need of fractionation plasma [1]. IVIGs are used to protect immunodeficient patients from recurrent bacterial or viral infections [2,3] and to treat an increasing number of autoimmune, inflammatory [4], and neurological [5] disorders. Like other biological products (e.g., a wide range of human plasma derived proteins, monoclonal antibodies, and recombinant proteins) produced from human or animal sources or using mammalian cells, IVIGs must be subjected to viral inactivation and/or removal procedures to ensure its viral safety for therapeutic use in humans [6].

Solvent/Detergent (S/D) is a robust, well established viral inactivation treatment applied to numerous biological products on the market [7,8], including IVIG. The S/D viral inactivation procedures often involve incubating protein solutions for 1 to 6 h in the presence of 0.3 to 1% of an organic solvent, typically tri-*n*-butyl phosphate (TnBP), and 0.2 to 1% of one or several detergents, usually

Tween 80 and (increasingly) Triton X 100 [9,10]. More recently, the application of Triton X 45 and Tween 20 combined with TnBP has also been described for the viral inactivation of plasma, cryo precipitate, and factor VIII [11–13].

One of the most crucial difficulties associated with S/D treatment is the removal of the solvent and detergent subsequent to the viral inactivation step. Three distinct procedures have been described for the removal of the S/D chemicals. One approach is to make use of ion exchangers or affinity resins in chromatographic processes designed to eliminate the S/D agents in the flow through (unadsorbed) fraction while target proteins are bound and then selectively eluted from the chromatographic material [14]. Other methods include phase extractions using vegetable oil typically soybean or castor oil followed by decantation to separate the oily and aqueous phases and to deplete the proteins from both TnBP and some oil soluble detergents such as Triton X 45 [7,11,12]. Finally, hydrophobic interaction chromatography (HIC) may be used to capture the S/D agents while the hydrophilic proteins are directly recovered in the chromatographic flow through [7]. This HIC process can be applied either directly on the S/D rich protein fraction or, as done for large pool plasma for transfusion [15,16] or fibrin sealant, after one oil extraction step introduced to reduce the concentration in S/D agents. The advantage of HIC is that it is fast and it induces minimal impact on protein composition.

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¹ Abbreviations used: IVIG, intravenous immunoglobulin G; S/D, solvent/detergent; TnBP, tri-*n*-butyl phosphate; HIC, hydrophobic interaction chromatography; LFB, Laboratoire Français du Fractionnement et des Biotechnologies; IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M; HIV, human immunodeficiency virus; HBV, hepatitis B virus; HCV, hepatitis C virus.

The goal of any procedure used for S/D removal is to guarantee that the residual amount of TnBP and detergent(s) is as low as possible in the final protein therapeutics more specifically, based on frequently accepted norms, less than 10 and 100 ppm of TnBP and detergent, respectively. The selection of an S/D removal procedure should also consider protein recovery, an aspect that is important when taking into account the scarcity of the starting material (e.g., plasma) as well as productivity and economical considerations. Historically, the HIC sorbents most often used for removal of S/D agents from therapeutic protein products have included silica based octadecyl (C18) materials [7,17] and SDR HyperD, a specially designed, three dimensional, cross linked hydrophobic acrylic polymer [18]. Both are regarded as effective materials to achieve satisfactory removal of S/D agents, but so far no direct comparison of the binding capacity of these sorbents for the solvent and detergent used for viral inactivation has been published. The aim of this study was to compare, for the first time, the efficiency of C18 and SDR HyperD packing materials to remove the S/D agents from one polyvalent plasma derived IVIG preparation that was experimentally subjected to viral inactivation by 1% TnBP combined with either 1% Triton X 100 or 1% Triton X 45.

Materials and methods

Experimental design

The experimental design is shown in Fig. 1 and described below.

Immunoglobulin G preparation

The IVIG preparation (10 g in 200 ml, freeze dried) was obtained from Laboratoire Français du Fractionnement et des Biotechnologies (LFB, Les Ulis, France). This product is manufactured following traditional plasma fractionation using a method derived from the well established Kistler Nitschmann ethanol fractionation process [19]. Fraction II containing purified immunoglobulin G (IgG) was further subjected to clarification, pepsin (pH 4.0) treatment [20,21], nanofiltration on 35 nm membranes, sterile filtration, and freeze drying. The product protein composition met all approved quality control specifications of the manufacturer. The total protein content of the preparation was 51 g/L. IgG, immunoglobulin A (IgA), and immunoglobulin M (IgM) contents were 48.4, 0.41, and 0.08 g/L, respectively. IgG monomers and dimers represented 98%, and IgG polymers and aggregates represented 0.4%. It was formulated at pH 6.6 and an osmolality of 397 mOsm/kg in the presence of sucrose.

S/D treatment

The freeze dried IVIG was reconstituted with 200 ml of water for injection (LFB) provided with the product. Complete reconstitution was obtained within 3 min at room temperature, and the clear IgG solution was used immediately after reconstitution without a filtration step. The IVIG preparation was aliquoted in three fractions of 65 ml each for the various S/D experiments described below. The following combinations of S/D were added at the specified final concentrations (v/v): (i) 1% TnBP (Prolabo, VWR, Fontenay sous bois, France) and 1% Triton X 100 (Merck, Darmstadt, Germany) and (ii) 1% TnBP and 1% Triton X 45 (Sigma Chemical, St. Louis, MO, USA). The IgG S/D mixtures were incubated for 2 h at 31 ± 0.5 °C under constant gentle stirring in a shaker incubator (Lab Therm LT W, Kühner, Switzerland).

S/D removal

On termination of the S/D incubation, each IgG S/D mixture was aliquoted in four fractions of 7 ml to be subjected, without additional processing or dilution, to chromatography with the SDR HyperD and C18 resins. The C18 bulk packing material used (batch 274 WAT020594) was obtained from Waters (Guyancourt, France). This material is commercialized as a dry powder. It has a porosity of 125 Å and a particle size of 55 to 105 µm. It was stored at room temperature prior to use according to the manufacturer's instructions. The SDR HyperD batch (20 033 0/G161 02) was obtained from Pall France BioSeptra (Cergy Saint Christophe, France). It is a sorbent composed of 40% polymer on a porous silica with a surface area of 200 m²/g and a particle size of 40 to 100 µm. The sorbent was stored at 4 °C until the experiment. Prior to use, the gel was washed with 5 volumes of 1 M sodium chloride and 20% ethanol and then with 5 volumes of 20 mM citrate buffer (pH 7.4).

Determination of weight/volume ratio of SDR HyperD

Here 50 ml of SDR HyperD (same batch number as used for the adsorption experiment) was equilibrated in 5 volumes of 20 mM citrate buffer (pH 7.4, 20 ml) of a 50% mixture of SDR HyperD, and citrate buffer was then poured into a graduated column. The volumes of sedimented gel were recorded after 15, 22, and 30 min. The whole mixture was then poured into a tared 100 ml sintered funnel (porosity no. 2, Belleville, Fontenay sous bois, France). Next, 3 volumes of 20%, 50%, and absolute ethanol (Carlo Erba, Val de Reuil, France) each was passed, respectively, onto the gel and drained under vacuum without complete drying. One additional wash with 3 volumes of absolute ethanol was carried out, followed by two washes using 100% acetone (Carlo Erba). The sintered funnel was dried completely, weighed (W0), and put in a 90 °C dry oven overnight. The sintered funnel was weighed

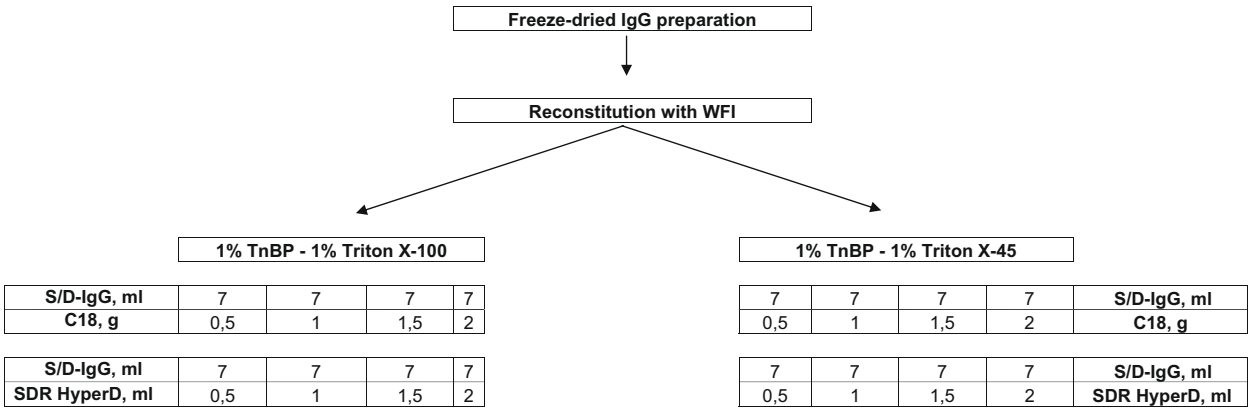


Fig. 1. Experimental design. For details, see Materials and methods.

immediately after cooling (W1) and 4 h later (W2). These experiments were performed in triplicate.

Adsorption tests

Increasing quantities of adsorption material (0.5, 1, 1.5, and 2 ml of SDR HyperD and 0.5, 1, 1.5, and 2 g of C18) were dispensed into Poly Prep chromatography columns (Bio Rad, Hercules, CA, USA). Then 7 ml of S/D IVIG was added and put in contact with SDR HyperD or C18 for 30 min at room temperature (25 °C) under mixing. The IVIG was recovered by gravity from the chromatographic column.

Quality control

Multiple 1 ml plasma samples were taken at all steps of the process, collected in closed plastic tubes (Becton Dickinson, Franklin Lakes, NJ, USA), kept on the bench at 22 to 25 °C, and frozen at 80 °C at the end of each run until analysis. The samples taken prior to the S/D treatment were used as negative controls for TnBP and Triton assays, and those after the S/D treatment were used as positive controls to confirm that the content of TnBP and Triton was within the expected concentration based on the respective additions of 1% (v/v, final concentration). Total protein content was determined by the Biuret method (Dialab, Wien, Austria) using albumin as a standard [12].

TnBP, Triton X 45, and Triton X 100 determination

TnBP determination. Content in TnBP was measured following modifications of the methods developed for determination in plasma and other plasma products described previously [12,22]. Perchloric acid (0.125 ml of 1.5 N) was added to the IVIG samples (1 ml), vortexed, and incubated in a water bath for 15 min at 37 °C. An ice cooled ethanol/chloroform mixture was added to the IVIG at a ratio of 1:0.092:1 ml, respectively, and incubated for 2 h at 20 °C. Tubes were centrifuged at 1200g for 30 min, the supernatant (S) was carefully transferred into clean tubes, and the TnBP residue was extracted three times with 2 ml each of *n* hexane. After the addition of hexane, the tubes were shaken vigorously and then allowed to settle. The upper hexane layer was carefully collected with a Pasteur pipette, without disturbing the intermediate layer, and was transferred into a clean tube. The hexane fractions were pooled (S₁). The precipitate (P) was extracted three times by adding 2 ml of *n* hexane and mixed thoroughly for 2 min. Samples were centrifuged (955g, 30 min, room temperature). Also, these hexane fractions were separated by centrifugation and pooled (S₂). The extracts from both the supernatants (S₁) and precipitates (S₂) were combined and dried under a stream of air or nitrogen. Then 100 to 200 µl of hexane was added, and the walls of the tubes were carefully rinsed and transferred into Eppendorf tubes (Eppendorf AG, Hamburg, Germany). This process was repeated five times to ensure that the residues of TnBP were transferred quantitatively. The volume in each Eppendorf tube was adjusted to 1 ml. The TnBP content was determined by injecting 2 µl of the extracted solution online into a gas chromatograph (SRI 8610C, SRI, Torrance, CA, USA) equipped with a flame ionization detector using nitrogen at 40 ml/min, 200 °C, and a Chromosorb WHP column (2 m length, 2 mm i.d., liquid phase FFAP, mesh range 80–100). The recovery of the TnBP extraction procedure was between 90 and 95%, and the limit of detection was less than 10 ppm.

Triton X 45 and Triton X 100 determination. IgG samples (1 ml) were extracted with ice cooled ethanol (2 ml) and chloroform (0.092 ml), incubated for 2 h at 20 °C, and centrifuged at 1200g for 30 min. Then 2 ml of ice cooled ethanol was added to the precipitates, mixed, shaken vigorously, and centrifuged for 30 min at 955g. The extraction of the precipitates was repeated five times,

and the supernatants were combined, concentrated to 1 ml, and kept at 8 °C. Samples were analyzed by high performance liquid chromatography (Gilson, Middletown, WI, USA) equipped with a binary pump and UV/VIS Detector 155 at 225 nm. For Triton X 100 and Triton X 45 determination, a Lichrosphere RP C18 5 column (125 × 4.6 mm, CS Chromatographie Service GmbH, Langerwehe, Germany) was used under isocratic conditions using a mobile phase of 85% methanol at a flow rate of 1.5 ml/min after a modification of the method described previously [12]. The run time was 10 min. A standard curve established with five distinct concentrations was drawn to express the Triton content in parts per million (ppm). The detection limit was less than 2 ppm.

Results and discussion

Plasma derived IVIGs are classified as essential medicinal products by the World Health Organization, illustrating the role they play in human health. Therefore, their viral safety is also of paramount importance. In spite of increasingly efficient and extensive testing procedures to detect the presence of viruses in individual blood and plasma donations, plasma pool for fractionation can still be contaminated by viruses due to donations during the window period, human errors, or the emergence of new viruses. Thus, viral inactivation and removal methods play a crucial role in the viral safety of these products [23]. Among the various viral inactivation and removal procedures available, the S/D treatment is recognized as a very effective way to ensure the inactivation of lipid enveloped viruses such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) [8,23,24]. However, long term shortages of plasma derived IVIGs are expected at the global level [1,25]; thus, it is important that viral inactivation procedures have a minimal impact on protein recovery. With these concerns in mind, we compared the capacities of C18 and SDR HyperD materials to remove S/D agents from IVIGs.

Determination of weight/volume ratio of SDR HyperD

Because one sorbent (C18) is available as a dry powder, whereas the other is commercialized as a wet packing material, the weight/volume ratio of the SDR HyperD gel was first determined (Table 1) to make a comparison with the S/D binding properties more objective. Data showed that the gel was sedimented with no significant variations in volume at 15 min (V15) compared with longer sedimentation periods (V22 and V30). Complete drying was achieved after a 90 °C overnight incubation, allowing reliable dry weight measurement (W1) to be made. The mean W0 (g)/V15 (ml) ratio was 0.44.

Adsorption capacity of C18 and SDR HyperD for TnBP, Triton X 100, and Triton X 45

After reconstitution with water for injection, the purified IVIG presented as a clear, particle free solution. The IVIG treated by the TnBP Triton X 100 combination developed a slight opalescence, but it was apparently less than what was reported by others using another IVIG preparation [26]. The IVIG virally inactivated by TnBP Triton X 45 was clear and particle free. All IVIG samples were treated, without dilution or filtration, at various ratios of hydrophobic sorbent materials. The residual amount of TnBP in all S/D treated IVIG samples was less than 10 ppm (not shown), even at the lowest C18 and SDR HyperD ratios (0.5 g and 0.5 ml, respectively, for 7 ml of IVIG). Considering that the IVIG contained 10 mg of TnBP/ml, this indicates that the binding capacities for TnBP were greater than 140 mg/g of C18 and greater than 140 mg/ml of SDR HyperD, corresponding to more than 318 mg/g

Table 1

Dry weight/volume (g/ml) ratios of SDR HyperD at various sedimentation times.

	Assay 1			Assay 2			Assay 3		
	15 min 10.5 ml	22 min 10.5 ml	30 min 10.5 ml	15 min 11.0 ml	22 min 10.9 ml	30 min 10.9 ml	15 min 10.5 ml	22 min 10.5 ml	30 min 10.5 ml
Weight W0 (g)	4.8			4.9			4.9		
Weight W1 (g)	4.6			4.7			4.7		
Weight W2 (g)	4.6			4.7			4.7		
Weight (g)/Volume (ml) at T1	0.44	0.44	0.44	0.43	0.43	0.43	0.45	0.45	0.45

of dry sorbent. TnBP removal efficiencies were higher than 99.5% with both sorbents. The residual amounts of Triton X 45 and Triton X 100 are shown in Table 2. Triton X 45 was detected only in the IVIG samples treated by the lowest ratio of C18 and SDR HyperD and was less than 2 ppm for all other conditions. Binding capacity of Triton X 45 was in excess of 70 mg/g of C18. It was in excess of 70 mg/ml of SDR HyperD, equivalent to more than 159 mg/g of dry SDR HyperD. Removal efficiency for Triton X 45 was higher than 99.9% under these experimental conditions. Good Triton X 100 binding capacity, although less than that observed for Triton X 45, was found with both packing materials, with all IVIG breakthrough samples containing less than 100 ppm. However, residual amounts were close to 50 ppm when using the lower ratios of C18 (0.5 g/7 ml) and SDR HyperD (0.5 ml/7 ml). Residual Triton X 100 gradually decreased to approximately 19 and 7 ppm when the quantities of SDR HyperD were increased to 1.0 and 1.5 ml/7 ml of IVIG, respectively, corresponding to a binding capacity, under these last conditions, of approximately 106 mg of Triton X 100/g of dry SDR HyperD. The residual amount of Triton X 100 in the IVIG breakthrough samples decreased to approximately 30 ppm at the highest ratio of C18 used (2 g/7 ml) but remained higher than when using SDR HyperD. Removal capacities of Triton X 100 at the ratios evaluated were higher than 99%.

Data show that both C18 and SDR HyperD hydrophobic sorbents ensure efficient removal of TnBP, Triton X 100, and Triton X 45. The respective binding capacities found here were slightly higher than those reported for SDR HyperD using crude plasma or other plasma derived preparations [18]. This can most likely be explained by the facts that (i) the IVIG used here was highly purified and essentially free of plasma small sized hydrophobic

or amphiphilic contaminants, (ii) the residence time was 30 min instead of 16 min, and (iii) a batch adsorption procedure was used instead of a column adsorption procedure. The capacity was also higher than that shown for Amberlite XAD 7, a mixed mode sorbent for which 1 ml of resin could bind only 2.8 mg of TnBP and 9 mg of Triton X 100 [26]. The high binding capacity of the SDR HyperD may result from the fact that the silica and the hydrophobic polymer, conferring a mixed mode structure, exert a distinct role in the adsorption of Triton and TnBP, respectively, and contribute to the high capacity per milliliter for both compounds. Previous studies [18] showed that large surface area of silica moiety favored Triton X 100 adsorption, whereas TnBP binding was increased with higher hydrophobic polymer content. In addition, variations in the polymer content between 20 and 40% were not found to influence the sorption capacity for Triton X 100 [18]. It is also worth noting that 1 g of C18 silica packing is equivalent to 2 ml of chromatographic packing, implying that packed bed column should have twice the volume of that used for SDR HyperD to achieve similar S/D removal. It should also be emphasized that the optimal ratios of IVIG material to HIC sorbents found in this study are valid for the specific IgG product used. It is possible that slightly different ratios would need to be used with other IVIG preparations or intermediates, depending on their content in hydrophobic compounds such as lipids.

Protein recovery

IgG recovery was assessed by measurement of total protein in accordance with the Monograph for IVIG in the European Pharmacopoeia, which does not include an assay for IgG. The total protein

Table 2

Residual contents (ppm) of TnBP, Triton X-45, and Triton X-100 and protein recovery in S/D-IVIG samples subjected to SDR HyperD or C18 chromatography.

Quantity of HIC material (ml or g)	SDR HyperD			C18		
	TnBP–Triton X-100 treatment			TnBP–Triton X-45 treatment		
	Residual TnBP (ppm)	Residual Triton X-100 (ppm)	Protein recovery (%)	Residual TnBP (ppm)	Residual Triton X-100 (ppm)	Protein recovery (%)
0.5	<2	52.78	104	<2	41.13	111
1	<2	19.35	101	<2	35.43	106
1.5	<2	7.28	100	<2	40.65	109
2	<2	7.05	92	<2	29.70	112
	TnBP–Triton X-45 treatment			TnBP–Tween 80 treatment		
	Residual TnBP (ppm)	Residual Triton X-45 (ppm)	Protein recovery (%)	Residual TnBP (ppm)	Residual Tween 80 (ppm)	Protein recovery (%)
0.5	<2	2.15	102	<2	3.86	93
1	<2	<2	104	<2	<2	98
1.5	<2	<2	94	<2	<2	95
2	<2	<2	99	<2	<2	102
0.5	<2	–	104	<2	–	102
1	<2	–	101	<2	–	104
1.5	<2	–	102	<2	–	95
2	<2	–	101	<2	–	103

Note. Protein recovery is equivalent to IgG recovery.

recoveries of the various S/D treated IVIGs after phase adsorption with SDR HyperD and C18 are presented in Table 2. Good recovery of protein was achieved with both sorbents regardless of the type of S/D treatment applied. Up to 700 mg of protein could be treated per milliliter or gram of sorbent. The apparent slightly greater protein content found in the TnBP Triton X 100 IgG samples after C18 chromatography is thought to be an artifact encountered in the colorimetric assay used to assess protein content due to the slight opalescence of the samples. The wet SDR HyperD sorbent led to a slight dilution of the samples to a range of 43 to 47 g of protein/L more noticeable, as expected, at the higher ratios of sorbents (1.5 and 2.0 ml/7 ml of IgG). However, because manufacturing processes usually include concentration and dialysis steps prior to final formulation, this is not considered to be a significant drawback.

It is also important to ensure that the procedure does not induce an alteration of the IgG molecules. A slight increase in IgG polymers from approximately 2 to 3% was found in a 0.3% TnBP 1% Triton X 100 treated IVIG chromatographed on Amberlite XAD 7 [26], but this slight increase may be due to the small scale ultrafiltration step that was also performed on the product. Currently used large scale S/D treatment and removal methods have been shown to have a good safety profile and not to induce an alteration on the Fc function or an increase in the content of polymers of several IVIG preparations [27–30].

Conclusion

This study has demonstrated that both SDR HyperD and C18 chromatographic packings can efficiently remove TnBP and various detergents used for the viral inactivation of human immunoglobulin preparations without impairing IVIG recovery. The mixed mode structure of the SDR HyperD sorbent appears to confer a global binding capacity expressed per gram of dry sorbent significantly greater than that of the C18 material, conferring a potential advantage because the column size can be reduced, leading to production cost reduction.

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