ELSEVIER

Journal of Chromatography B, 664 (1995) 119–125

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Specific sorbent to remove solvent–detergent mixtures from virus-inactivated biological fluids

L. Guerrier[a,*], I. Flayeux[a], E. Boschetti[a], M. Burnouf Radosevich[b]

[a]*BioSepra SA, 35 avenue Jean Jaurès, 92395 Villeneuve la Garenne, France*

[b]*CRTS-Lille, Laboratoire Français du Fractionnement et Biotechnologies (LFB-Lille), 59 rue Trévise, 59011 Lille, France*

Abstract

A method involving a sorbent designed to capture specifically virus-inactivating solvent–detergent mixtures is described. Its specificity allows the asdsorption of these undesirable chemicals with a high capacity in order to treat large amounts of inactivated biological fluids in small-sized columns. Typically, the volume of sorbent that can be used repeatedly is between one quarter and one tenth of the sample volume to be treated. Cleaning and sanitization can be done by classical methods while strong oxidizing agents can also be used to sterilize the sorbent.

1. Introduction

It is well established that one or more inactivation steps are necessary to diminish the risk of virus transmission from therapeutic plasma protein concentrates. The most commonly used methods for the viral inactivation of human plasma products include dry heat treatment [1,2], pasteurization [3,4], and ultraviolet irradiation alone [5] or in association with β-propiolactone [6]. General reviews of virus inactivations have been published [7,8]. Organic solvents in the presence of surfactants have also been described as an efficient way to inactivate enveloped viruses [9,10]. Solvent–detergent mixtures can reduce the infectivity of viruses by several orders of magnitude. Their effectiveness is explained by their properties to disassemble the virus envelope with consequent alteration of viral infectivity.

The use of solvent–detergent mixtures implies first the addition of these molecules to the biological fluid to be inactivated, followed by their removal in a selective step [11]. The elimination of solvent and detergent from virus-inactivated biological fluids is a real technical challenge and a number of techniques have been developed based on differences in density, sorbent partitioning, size exclusion and affinity-based interaction.

Batch extraction with vegetables oils has been reported [12]. Oil is thus added to a biological fluid containing the virus-inactivating agents, the mixture is swirled and then allowed to settle to extract the lipid-like molecules in the upper oil phase, then the latter is removed by siphoning and/or centrifugation.

Based on the molecular mass difference, gel permeation has also been described to eliminate

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00363-7

some solvent–detergent mixtures from biological fluids, taking advantage of the large size of proteins compared with inactivating agents [13]. This operation is accomplished on a chromatographic column containing a molecular sieve support with an M_r exclusion limit of 25 000.

More recently, Horowitz et al. [14] proposed the use of reversed-phase chromatography using C_{18}-grafted silica to remove solvent–detergent mixtures by phase partitioning on a solid material.

In this paper, we describe the application and performance of a specific sorbent for solvent–detergent removal, based on a mixed-mode adsorption associated with a molecular exclusion effect.

2. Experimental

2.1. Chemicals

Solvent–detergent removal (SDR) HyperD sorbents were supplied by BioSepra (Villeneuve la Garenne, France). This chromatographic packing was made of silica beads in which the pore volume was filled with a three-dimensional cross-linked hydrophobic acrylic polymer.

Tri-*n*-butylphosphate (TBP), Triton X-100, buffer components and analytical-reagent grade chemicals were obtained from Aldrich Europe (Brussels, Belgium). All biological materials such as antithrombin III (ATIII), immunoglobulins G (IgG) and bovine serum were purchased from Sigma (St. Louis, MO, USA).

2.2. Chemical treatment of biological fluids with TBP and Triton X-100

The treatment of solutions of biological materials such as IgG, ATIII and serum was carried out as a model to determine the efficiency of the investigated sorbent in removing the inactivating agents. The method used was taken from Prince et al. [9] and was applied as follows. A 250-μl volume of TBP and 500 μl of Triton X-100 were added to 50 ml of physiological protein solution (the protein concentration was 10 mg/ml for IgG and ATIII and 40 mg/ml for bovine serum) and then stirred gently for 5 h at room temperature. The final solution became cloudy owing to the formation of a microemulsion of TBP in the presence of Triton X-100. This solution was then directly processed.

2.3. Chromatographic method for solvent–detergent removal

SDR-HyperD stored as an aqueous suspension in 1 *M* sodium chloride and 20% ethanol was classically packed in small-scale laboratory glass columns and equilibrated in physiological phosphate buffer. The protein solutions containing TBP–Triton X-100 were in phosphate-buffered physiological saline (PBS) before injection into the column at a flow-rate of 150 cm/h. Different loaded volumes were tested. The collected protein fractions were then checked for their residual content of TBP and Triton X-100. Other experiments using physiological solutions of TBP and Triton X-100 without protein were also performed at different flow-rates. The columns were then washed with the same physiological buffer, regenerated by washing with ethanol and 2-propanol, then re-equilibrated.

2.4. Assays for TBP and Triton X-100

TBP was extracted from the protein solution and assessed by a gas chromatographic method as described previously [15].

According to the HPLC assay described by Strancar et al. [16], residual amounts of Triton X-100 were measured by using a Merck (Darmstadt, Germany) L6200 HPLC system coupled with a Vydac C4-RPC analytical column. A 1-ml volume of protein solution containing Triton X-100 was added to 1.5 ml of acetone to precipitate the proteins and 20 μl of clear supernatant were injected into a reversed-phase column (150 × 4.6 mm I.D.). An acetonitrile gradient in 0.1% aqueous trifluoroacetic acid solution was used to elute Triton X-100, which was detected by measuring the UV absorbance at 280 nm. A calibration graph obtained with known amounts of Triton X-100 was established as a reference

for further calculations. The detection limit of the assay was estimated to be 10 ppm, which corresponded to a UV absorbance of $6 \cdot 10^{-4}$ AUFS.

3. Results and discussion

Trials made with SDR-HyperD sorbents having different silica surface areas and hydrophobic polymer contents indicated that a large surface area of silica moiety was necessary to obtain high sorption capacity for Triton X-100. On the other hand, the sorption capacity for TBP increased slightly when the hydrophobic polymer content increased (Fig. 1).

An increase in the concentration of three-dimensional cross-linked polymer generally reduces its exclusion limit. This phenomenon resulted in a dramatic decrease in protein diffusion into the bead network and reduced the contact of proteins with the polymer itself, thus reducing the risk of non-specific binding and of protein denaturation.

Gel permeation experiments with standard molecular mass compounds, carried out to define the exclusion limit of the sorbent, showed that proteins with a molecular mass higher than 10 000 passed into the void volume. This was obtained with a sorbent composed of 40% of polymer on a porous silica with a surface area of around 200 m^2/g.

This particular behaviour demonstrated that both the silica and the hydrophobic polymer play an active and distinct role in the adsorption of Triton X-100 and TBP, respectively. Adsorption of Triton X-100 was proportional to the silica surface area; each gram of 200 m^2/g porous silica adsorbed 120 mg of Triton X-100 whereas 75 and 25 m^2/g silicas showed lower adsorption capacities of 44 and 22 mg/g, respectively. It is worth noting that 1 g of porous silica corresponds to 2 ml of chromatographic packing. This is in agreement with the formation of hydrogen bonds between the polyoxyethylene chains of

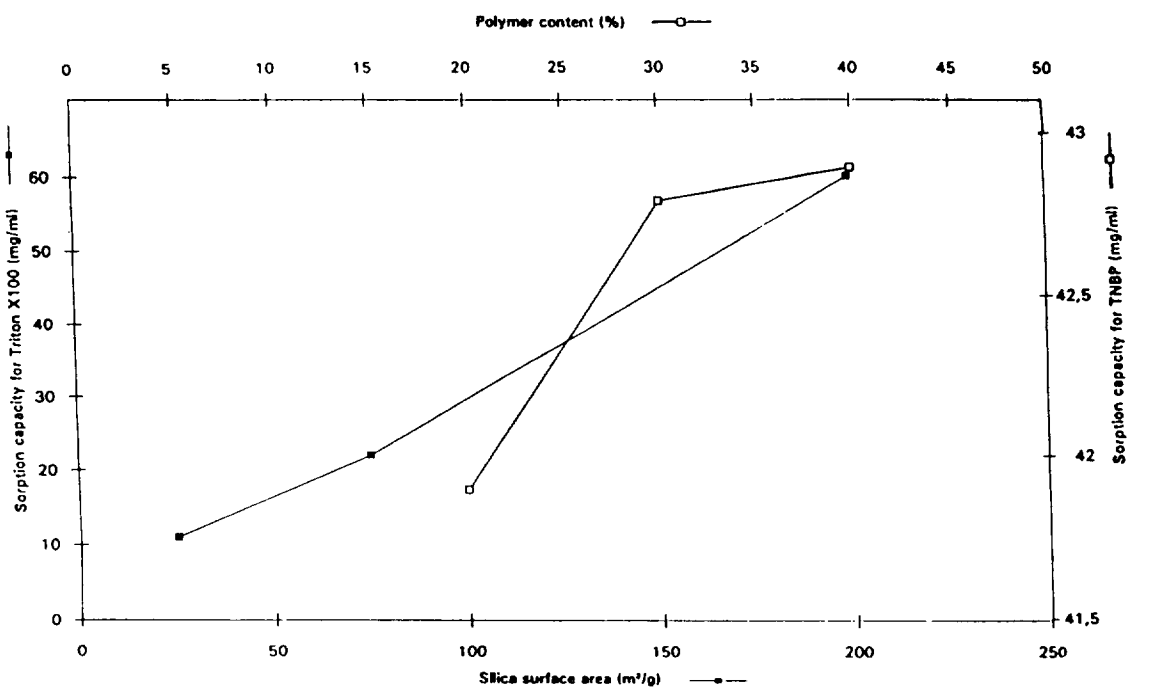

Fig. 1. Sorption capacity of SDR-HyperD for (■) Triton X-100 and (□) TBP as a function of surface area of silica and polymer content, respectively. Linear velocity, 150 cm/h.

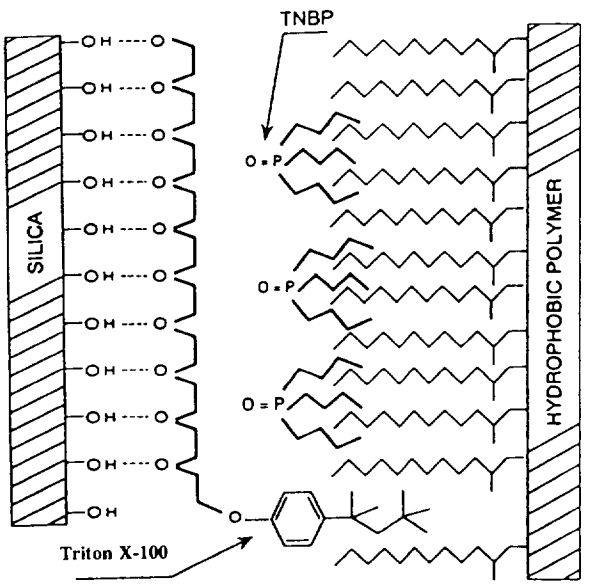

Fig. 2. Schematic interaction mechanism of Triton X-100 and TBP on SDR-HyperD. Triton X-100 interacts both with the silica surface (formation of hydrogen bonds between the silanols and the polyoxyethylene chain) and with the hydrophobic polymer moiety; TBP interacts only with the hydrophobic polymer of the sorbent.

Triton X-100 and the silanol groups present on the silica surface [17]. On the other hand, the amount of organic polymer within the silica pores influenced the sorption capacity for TBP. Polymer concentrations of 40%, 30% and 20% showed, under constant overloading conditions, residual amounts of TBP in serum of 10, 20 and 120 ppm, respectively. Variations in the polymer content did not influence the sorption capacity for Triton X-100. According to the observed behaviour of both components, the interaction mechanism in Fig. 2 is proposed.

Using bovine serum treated with TBP and Triton X-100, a number of experiments were performed in order to determine the influence of operating parameters on the depletion efficiency. It appears that the loading volume (Fig. 3) influences the efficiency of removal of the detergent. Using a constant linear velocity of 150 cm/h and a 10-cm column, a load of two column volumes resulted in almost total elimination of Triton X-100 (99.5%). The depletion efficiency for serum loadings of four and eight column volumes was 98% and 80%, respectively. The

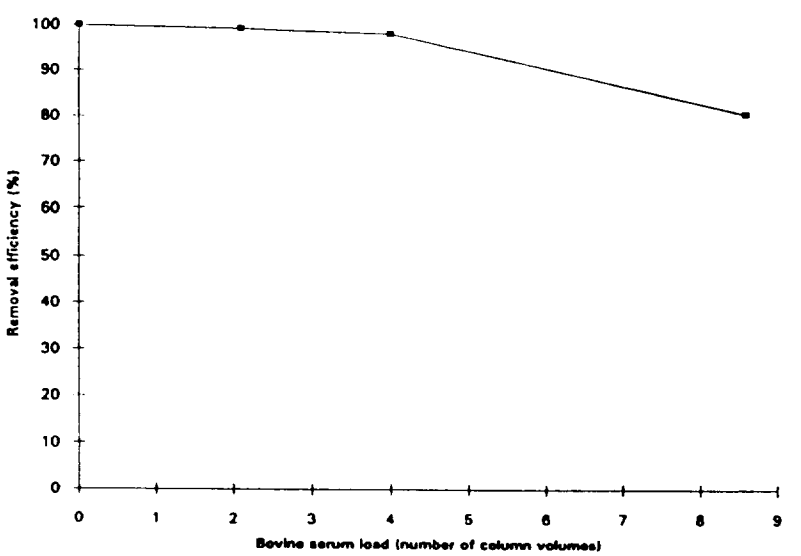

Fig. 3. Influence of the sample load (bovine serum) on the removal efficiency of SDR-HyperD for Triton X-100. Experiments were made on a 10-cm column at a linear velocity of 150 cm/h, corresponding to a residence time of 4 min.

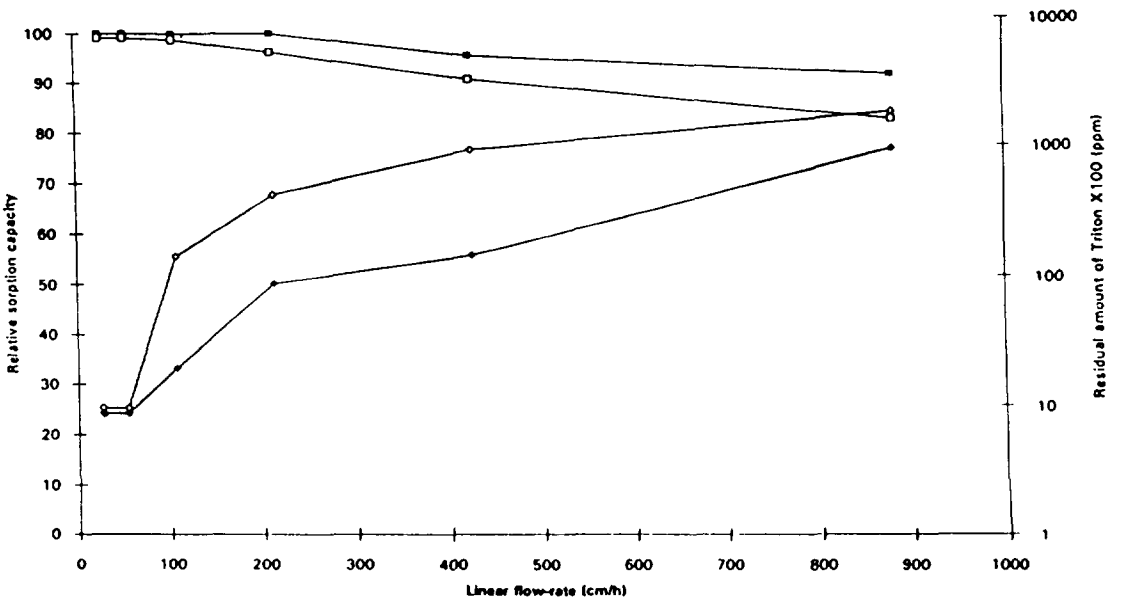

Fig. 4. Influence of linear velocity on (■, □) sorption capacity of SDR-HyperD for Triton X-100 and (◆, ◇) removal efficiency expressed as a residual amount in ppm after the chromatography of bovine serum. Experiments were carried out on two columns 220 mm (closed symbols) and 115 mm long (open symbols). Sample load, 4.3 column volumes.

linear velocity also affected the removal efficiency, as shown in Fig. 4. For a 22-cm column using a constant load of 4.3 column volumes, the sorption capacity for Triton X-100 decreased by 0.77% and 9.2% on increasing the linear velocity from 125 to 200 cm/h and from 125 to 880 cm/h, respectively. Residual amounts of Triton X-100 from the column effluents were undetectable at linear velocities less than 100 cm/h but increased from 100 and to 940 ppm at 200 and 880 cm/h, respectively. For a constant load of 4.3 column volumes, a residence time of about 16 min seems sufficient to afford the maximum possible removal efficiency.

The initial buffer conditions did not play an important role in the sorption capacity of the column as long as the pH was maintained at 7.4, corresponding to physiological conditions. Citrate, phosphate and acetate buffers (20 m*M*) were used in this respect with bovine serum with a load of eight column volumes and a linear velocity of 150 cm/h.

Non-specific binding tests for SDR-HyperD made with human albumin (10 mg/ml, in PBS buffer) injected in a large amount (ten column volumes), revealed the adsorption of 0.2–0.8 mg of albumin per ml of packing material. This represents a minimum protein loss as 200–500 mg of proteins can be treated per ml of SDR-HyperD sorbent. Non-specifically bound proteins can be removed with ethylene glycol followed by ethanol-enriched buffers.

Other experiments with the SDR-HyperD to eliminate Triton X-100 and TBP from virally inactivated ATIII and IgG solutions demonstrated that it is possible to treat a sample volume as high as 3.6 times the column volume with a residence time of only 4 min. As shown in Table 1, the elimination of both virus-sterilizing agents under these conditions was 100%.

When using bovine serum under the same conditions, the removal efficiency for TBP and Triton X-100 was 99.92% and 95.20%, respectively. The better solvent–detergent depletion observed with ATIII and IgG was probably due to the absence of small-sized hydrophobic or amphiphilic components present in serum that may compete with virus-stabilizing agents for the sorption sites on the SDR-HyperD sorbent. A larger amount of sorbent or a smaller load

Table 1
Triton X-100 and TBP removal experiments with SDR-HyperD from protein solutions

Compound	Parameter	IgG	ATIII	Bovine serum
Triton X-100	Initial concentration (mg/ml)	10	10	10
	Final concentration (ppm)	<10	<10	340[a]
	Removal efficiency (%)	100	100	95.2
TBP	Initial concentration (mg/ml)	5	5	5
	Final concentration (ppm)	<0.4	<0.4	3.8
	Removal efficiency (%)	100	100	99.92

Column, 10 cm × 0.33 cm I.D.; loading volume, 8 column volumes; linear velocity 150 cm/h (residence time 4 min).
[a] Overloading conditions.

enhances the removal efficiency of the chromatographic system to a quantitative level (Table 2).

Columns can only be reused if solvents and detergents are washed out after each cycle. Owing to the nature of these chemicals and the physico-chemical interactions, proper regeneration of the column can only be accomplished by the sequential use of solutions. An ethanol–water mixture (50:50) followed by pure ethanol, pure 2-propanol and then physiological buffers seem necessary to restore the initial properties of the column. As this type of column is intended to last for about 100 cycles, cleaning steps for removal of non-specifically adsorbed proteins have to be ensured using, for instance, concentrated solutions of urea or guanidine hydrochloride (8 and 6 *M*, respectively).

Effective disinfection operations are also of particular importance to eliminate residual nucleic acids from viruses, microbes or the biological fluid itself. For this purpose, diluted solutions of peracetic acid have been found to be very effective [18] without any risk of polymer degradation. A number of trials made in this respect using peracetic acid solutions in acetate buffer (pH 5) containing 20% ethanol demonstrated that after 100 cycles of peracetic acid treatment alternated with normal cycles in physiological buffer, the sorption capacity for Triton X-100 was 65.3 mg per ml of resin, compared with 66.7 mg before treatment.

Table 2
TBP and Triton X-100 removal efficiency (%) of SDR-HyperD from different biological preparations

Compound	Bovine serum	Foetal bovine serum	IgG	ATIII
Triton X-100	95.5	99.5	99.5	99.5
TBP	99.9	99.9	99.9	99.9

Sample load, 3.6 column volumes; linear velocity, 150 cm/h; column length, 100 mm.

Experiments to optimize the operating conditions (column geometry, residence time and load) and investigations on possible activation or inactivation of proteins from treated biological fluids are in progress.

In conclusion, an SDR-HyperD chromatographic packing has been investigated for its ability to remove efficiently TBP and Triton X-100 mixtures from virus-inactivated biological samples. A mixed-mode adsorption overlapping with a size-exclusion mechanism renders this sorbent very powerful and attractive to compete with existing classical methods, which are time consuming or of poor efficacy. The possibility of testing the SDR-HyperD sorbent with strong oxidizing chemicals, which is an unequivocal mode of sterilization, should stimulate research on applications in the field of human plasma fractionation and cell culture media.

References

[1] B. Hollinger, G. Dolana, W. Thomas and F. Gyorkey, *J. Infect. Dis.*, 150 (1985) 250.

[2] S.L. Dietrich, J.W. Mosley, J.M. Lusher, M.W. Hilgartner, E.A. Operskalsky, L. Habel, L.M. Aledort, G.F. Gjerset, M.A. Koerper, B.H. Lewis and C.H. Pegelow, *Vox Sang.*, 59 (1990) 129.
[3] S.S. Gellis, J.R. Neefe, J. Stokes, L.E. Strong, C.A. Janeway and G. Scatchard, *J. Clin. Invest.*, 27 (1947) 239.
[4] G. Dolana, D. Tse and W. Thomas, *J. Am. Soc. Hematol.*, 60 (1982) 2102.
[5] R. Murray, J.W. Oliphant and J.T. Tripp, *J. Am. Med. Assoc.*, 157 (1955) 8.
[6] W. Stephan and A.M. Prince, *Haemostasis*, 10 (1981) 67.
[7] T. Burnouf, *Biologicals*, 20 (1992) 91.
[8] J.-J. Morgenthaler, *Colloq. INSERM*, 227 (1993) 221.
[9] A.M. Prince, B. Horowitz and B. Brotman, *Vox Sang.*, 46 (1984) 36.
[10] *US Pat.*, 4 481 189 (1984) and 4 540 573 (1985).
[11] M.S. Horowitz, S.D. Bolmer and B. Horowitz, *Bioseparation*, 1 (1991) 409.
[12] *Eur. Pat.*, 0 239 859 (1987).
[13] B. Horowitz, M.E. Wiebe, A. Lippin and M.H. Stryker, *Transfusion*, 25 (1985) 516.
[14] B. Horowitz, R. Bonomo, A.M. Prince, S.N. Chin, B. Brotman and R.W. Shulman, *Blood*, 79 (1992) 826.
[15] C. Michalski, F. Ball, T. Burnouf and M. Goudemand, *Vox Sang.*, 55 (1988) 202.
[16] A. Strancar, P. Raspor, H. Schwinn, R. Schütz and D. Josic, *J. Chromatogr. A*, 658 (1994) 475.
[17] C.R. Hasting, J.M. Augl, S. Kapila and W.A. Aue, *J. Chromatogr.*, 87 (1973) 49.
[18] A. Jungbauer and H. Lettner, *Biopharm.*, June (1994) 64.