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Journal of Chromatography A, 852 (1999) 87–91

JOURNAL OF CHROMATOGRAPHY A

Short communication

Removal of detergent and solvent from solvent–detergent-treated immunoglobulins

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Abstract

The solvent–detergent (S/D) method was applied for inactivation of lipid-enveloped viruses during the production of immunoglobulins. Amberlite XAD-7 resin was used for removal of solvent (tri-*n*-butyl phosphate, TnBP) and detergent (Triton X-100) after the performed S/D inactivation procedure. The S/D reagents from the immunoglobulin preparation were adsorbed on Amberlite XAD-7, while immunoglobulins passed through the column and retained their biological activity. Using the method developed here, the final immunoglobulin preparation contains less than 1 ppm of Triton X-100 and less than 2 ppm TnBP. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Solvent–detergent method; Amberlite XAD-7 resin; Immunoglobulins; Triton X-100; Tributyl phosphate

1. Introduction

Despite the progress in donor selection and blood screening methods, some infectious viruses can still be transmitted by single-donor blood products and large-pool plasma derivatives. Human immunoglobulins as plasma derivatives, have been assumed to be one of the safest biological products. The immunoglobulin preparation obtained by the cold ethanol fractionation process carries no discernible risk of transmitting the human immunodeficiency virus (HIV) [1–3], but the transmission of hepatitis C by intravenous immunoglobulin preparations has been noted [4–6]. Therefore, virus inactivation plays a key role in the manufacturer's production of immunoglobulin preparations.

Most of the human pathogenic viruses found in blood have a lipid envelope (HIV, HBV, HCV). These viruses can be inactivated by the solvent–detergent (S/D) method developed by Horowitz et al. [7]. The virus inactivation was performed by tri-*n*-butyl phosphate (TnBP) as a solvent and Triton X-100 or Tween 80 as a detergent. The S/D substances responsible for viral inactivation must be removed from treated products before their clinical use. For elimination of solvent and detergent from virus inactivation biological fluids a number of techniques have been developed based on differences in density, sorbent partitioning, size-exclusion and affinity based interaction [8–11]. During the production of commercial immunoglobulin preparations, S/D substances are usually removed by extraction with castor-oil and by solid-phase extraction using reversed-phase support [12] or cation-exchange chromatography [13].

In this paper we described elimination of virus-inactivating reagents from immunoglobulins in one step using Amberlite XAD-7 resin. Amberlite XAD-7 has an aliphatic matrix consisting of polymethacrylates, it is of intermediate polarity, and can effectively adsorb substances up to a molecular mass of 45 000 by hydrophobic interactions. It was re-

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00178-8

ported earlier that Amberlite XAD-7 was used to remove cytokines and endotoxin from plasma [14,15]. The effect of plasma protein binding to the Amberlite XAD-7 was also investigated [16] as well as the application of different Amberlites (XAD-2, XAD-4, XAD-7) for extraction of leucotrienes from plasma samples [17,18]. In this paper we have shown that Amberlite XAD-7 under specified conditions adsorbed solvent and detergent, while immunoglobulins passed through the column without interaction.

2. Experimental

2.1. Chemicals

Triton X-100 was purchased from BDH (London, UK) and TnBP from Aldrich (Steinheim, Germany); Amberlite XAD-7 was supplied by Rohm and Haas (Philadelphia, PA, USA); Sephacryl S-300 HR was supplied by Pharmacia (Uppsala, Sweden).

All other chemicals were purchased from Merck (Darmstadt, Germany) or Serva (Heidelberg, Germany).

2.2. Methods

2.2.1. Solvent–detergent treatment of immunoglobulins

Freeze–dried immunoglobulins produced by cold ethanol fractionation [19] at the Institute of Immunology, Zagreb, Croatia, were dissolved in water for injection at pH 5.3 to 5.5 and filtered through a 2-μm filter. Then 0.3% (w/w) TnBP and 1% (w/w) Triton X-100 were added and gently mixed for 6 h at room temperature. The final solution became cloudy due to the formation of microemulsion of TnBP in the presence of Triton X-100. Taking into account high protein concentration (more than 100 mg ml^{-1}) and the turbidity of solution after S/D treatment, the immunoglobulin solution was diluted three times before further processing.

2.2.2. Chromatographic method for solvent–detergent removal

Removal of S/D reagents was carried out by an adsorption process on Amberlite XAD-7 resin. The resin was suspended and equilibrated in 0.01 *M* phosphate buffer, pH 7.1 containing 0.5 *M* NaCl. After being diluted three times in equilibrated buffer the sample was applied and subsequently washed with the same buffer until the absorbance at 280 nm was less than 0.05. The resin was then washed with 1.0 *M* NaCl in the same buffer to remove the rest of eventually bound non-specific proteins.

The adsorbed S/D reagents were then eluted with 96% (w/v) ethanol and the column was subsequently washed with water for injection and re-equilibrated. The sanitation of Amberlite XAD-7 resin was performed by washing with 0.5 *M* sodium hydroxide.

In order to determine the capacity of Amberlite XAD-7 for binding of Triton X-100 and TnBP, experiments on analytical columns (5×1.6 cm) have been performed. Different quantities of S/D-treated immunoglobulins (2.0, 2.5, 5.0 and 10.0 ml) after being diluted three times with column buffer, were applied to the columns which contained the same volume of Amberlite XAD-7 (~10 ml) and were eluted as already described. The collected fractions were checked for proteins, TnBP and Triton X-100 content. After definition of the capacity of Amberlite XAD-7 resin, further analytical experiments were performed with 7 ml of S/D-treated immunoglobulins, which were applied to approximately 40 ml of Amberlite XAD-7 (20×1.6 cm) and were eluted as previously described. Eluted fractions were pooled and examined for proteins, TnBP and Triton X-100. The fraction pool which contained immunoglobulins was not processed further because of the small amount eluted.

A pilot-scale experiment was performed with 2 l of S/D-treated immunoglobulins. After S/D treatment and dilution the sample was applied to approximately 11 l of Amberlite XAD-7 (glass column BPG 200/500, Pharmacia), equilibrated in the same buffer using the Bioprocess system. The flow-rate was 14 ml cm^{-2} h^{-1}. The resin was washed with the same buffer and the immunoglobulins were collected until absorbance at 280 nm was less then 0.1. The collected immunoglobulins pool was ultrafiltrated using Minisette Systems with three membranes of M_r 30 000 (Filtron, Northborough, MA, USA). When the volume of the immunoglobulin solution was about 1.5 l, diafiltration (using the water for injection) was started. The process was terminated by adding the

solid glycine to final concentration of 0.3 *M*. The pH of immunoglobulins was adjusted to 6.6–6.7 and finally, immunoglobulins were filtered through a 0.22-μm filter.

2.2.3. Analytical techniques

Protein concentration was determined by the Biuret method [20], using immunoglobulin as a standard. TnBP was extracted from immunoglobulin solution and assayed by gas chromatography, as described previously [21]. Residual Triton X-100 was determined by column switching high-performance liquid chromatography (HPLC) according to the assay described by Štrancar et al. [22]. Gel chromatography on Sephacryl S-300 HR was applied for quantitation of immunoglobulin components. The peak areas for monomers, dimers and polymers were quantitatively expressed by computer integration. The content of tetanus and hepatitis A antibodies were determined by enzyme-linked immunosorbent assay (ELISA; Institute of Immunology, Zagreb, Croatia and Organon Teknika Boxtel, The Netherlands). IgG, IgA and IgM were measured by radial immunodifusion technique using specific anti-human serum (Institute of Immunology, Zagreb, Croatia).

3. Results and discussion

The adsorption capacity of S/D reagents on Amberlite XAD-7 resin was determined and results are given in Table 1. In all samples (2–10 ml), from S/D-inactivated immunoglobulins which passed through the resin, more than 90% of Triton X-100 and TnBP were bound. Under the described experimental conditions, 1 ml of Amberlite XAD-7 resin adsorbed more than 9 mg of Triton X-100 and more than 2.8 mg of TnBP. As the remaining residual amounts of S/D reagents in final immunoglobulins should be very low because of the safety reason, we have chosen conditions where most of the S/D reagents from the applied sample were adsorbed on the resin. It was achieved when 2 ml of S/D-treated immunoglobulins was applied on 10 ml of Amberlite XAD-7 resin. Thus, further experiments are based on adsorption capacity of approximately 2 mg Triton X-100 and 0.6 mg TnBP per 1 ml Amberlite XAD-7. Under these conditions we performed three separate analytical experiments for removal of S/D reagents from 7 ml S/D-treated immunoglobulins using approximately 40 ml Amberlite XAD-7 (20×1.6 cm). A typical elution profile of one these experiments is shown in Fig. 1. Most of the immunoglobulins were collected in pool fractions 1–50 (72%) (Fig. 1), but because of the low yield, the column was further washed with the starting buffer. S/D reagents were then desorbed using 96% (w/v) ethanol and two peaks were detected (Fig. 1, E3). In the first peak, 90% of Triton X-100 and 94.2% of TnBP were eluted while the second peak contained 9.2% of Triton X-100, 3.4% of TnBP and certain quantity of denatured immunoglobulins which were probably non-specifically bound to the resin. In pool fractions which contained immunoglobulins, Triton X-100 and TnBP were not detected.

After these experiments, the pilot-scale experiment was performed on a BioProcess chromatographic system. The solution of the S/D-treated immunoglobulins (after removal of S/D reagents by ad-

Table 1
Triton X-100 and TnBP removal from S/D treated immunoglobulins solution utilizing Amberlite XAD-7 – the determination of capacity[a]

S/D-treated immunoglobulins	Proteins			Triton X-100			TnBP		
Volume applied (ml)	Applied (mg)	Eluted (mg)	Recovery (%)	Applied (mg)	Eluted (mg)	Recovery (%)	Applied (mg)	Eluted (mg)	Recovery (%)
10	1360	1088	88	100	94.4	94.4	30	28	93.3
5	680	620	91	50	48.0	96.0	15	14.2	94.6
2.5	340	306	90	25	24.5	98.0	7.5	7.3	97.3
2	272	250	92	20	19.8	99.0	6.0	5.9	98.3

[a] 2.0, 2.5, 5.0 and 10.0 ml S/D-treated immunoglobulins after dilution were applied separately to columns (5×1.6 cm, 10 ml bed volume) and eluted (see Experimental).

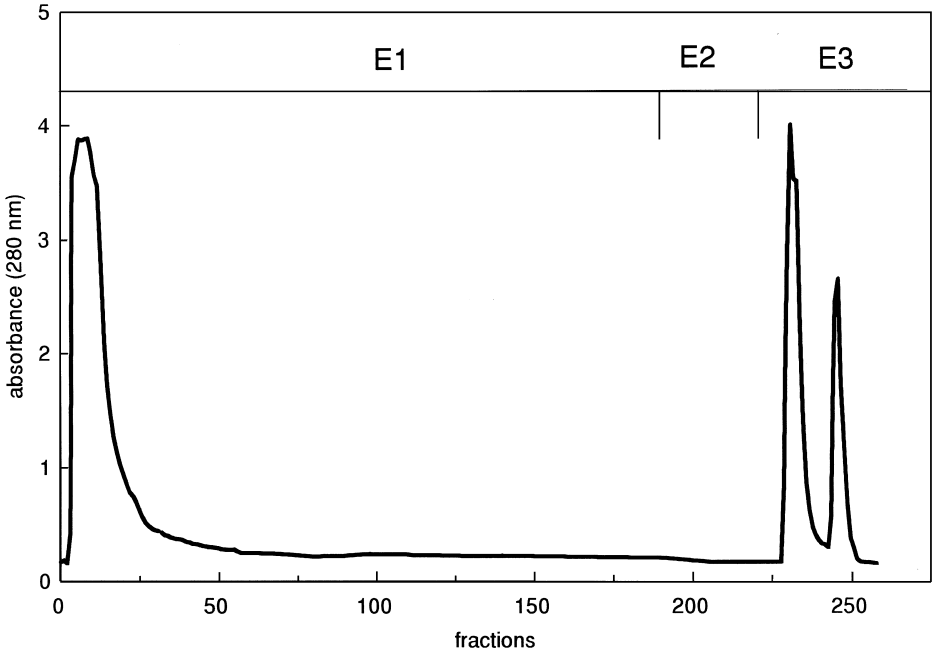

Fig. 1. Removal of S/D reagents from treated immunoglobulins (7 ml) using Amberlite XAD-7 in an analytical experiment. Chromatographic conditions: column 20×1.6 cm (approximately 40 ml bed volume), flow-rate 28 ml cm^{-2} h^{-1}, fractions 7 ml; eluents: 0.5 *M* NaCl in 0.01 *M* phosphate buffer (E1), 1.0 *M* NaCl in 0.01 *M* phosphate buffer (E2) and 96% (w/v) ethanol (E3). The fractions were pooled and checked for proteins, TnBP and Triton X-100. The first peak contained immunoglobulins while S/D reagents were eluted with eluent E3 in the other two peaks.

sorption on Amberlite XAD-7 resin), was ultrafiltrated using Minissete membrane cassettes. Glycine was added to the diafiltrated immunoglobulins, pH was adjusted and finally immunoglobulins were filtered through a 0.22-μm membrane. For immunoglobulins prepared in this way, residual TnBP and Triton X-100, proteins and antibody content were determined and compared with starting S/D non-treated immunoglobulins. These results are shown in Table 2. During this described manufacturing procedure about 35% of the starting proteins were lost. Apart from that, the loss of immunoglobulins of different classes or specific antibodies were not observed but slightly higher content of the polymers was observed (Table 2). The content of the Triton X-100 was under the detection limit when column-

Table 2
Production of immunoglobulins including S/D treatment[a]

Samples	Proteins (g)	Classes of immunoglobulins (%)			Polymers (%)	Antibody content (IU g^{-1} protein)		Triton X-100 (μg ml^{-1})	TnBP (μg ml^{-1})
		IgG	IgA	IgM		Anti-tetanus	Anti-hepatitis A		
Starting immunoglobulins									
	272±16	98.3±1	1.6±0.3	<0.1	2.1±0.2	485.7±22	785.7±30	10 000	3000
S/D-treated immunoglobulins									
1	178.9	98.0	1.9	<0.1	2.95	462.0	693.0	<1	0.4
2	184.1	97.5	2.4	<0.1	2.88	426.7	812.0	<1	1.6
3	171.9	98.7	1.2	<0.1	3.63	481.6	688.8	<1	0.1

[a] Three separate experiments were performed (see Experimental) and final products were compared with the starting immunoglobulins solution.

switching HPLC was used. The content of the TnBP was less than 2 μg ml^{-1} (Table 2). These values, especially for Triton X-100 were lower than literature data obtained with existing classical methods [8,9,11].

The application of Amberlite XAD-7 as a sorbent for S/D removal is probably based on a mixed-mode adsorption associated with a molecular exclusion effect. The interaction between Amberlite XAD-7 and S/D is apparently stronger than interaction between immunoglobulins and S/D which is why S/D can be separated from immunoglobulins. To reduced the risk of non-specific protein binding to Amberlite XAD-7 resin, the applied buffer contained 0.5 *M* NaCl. Similar experiments of S/D removal from biological fluids with the use of three-dimensional cross-linked hydrophobic acrylic polymers (SDR-HyperD) was described by Guerrier et al. [11]. Comparing with our results, the adsorption capacity of SDR-HyperD sorbent for TnBP is higher than that of Amberlite XAD-7 resin, but for Triton X-100 the situation is opposite. Other similar methods for removal of S/D reagents from biological fluids are based on reversed-phase liquid chromatography. Horowitz et al. [9] have shown that S/D reagents were effectively removed from treated human plasma by C_{18} Prep resin after previous extraction with soybean oil. One ml of C_{18} Prep resin effectively removed Triton X-100 and TnBP from 6 ml of treated plasma [9]. Obviously, capacity of C_{18} Prep resin is higher than that of Amberlite XAD-7, but residual contents of TnBP and Triton X-100 are lower when Amberlite XAD-7 was used, as Table 2 shows [9]. According to the obtained results and with regard to the low cost of Amberlite XAD-7 resin, further research about the application of this material in the field of the human plasma fractionation should be stimulated.

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