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Short communication

Ion-exchange chromatography separation of the detergent and the solvent from immunoglobulins after solvent–detergent treatment

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Abstract

For inactivation of lipid-enveloped viruses during the immunoglobulin production, the solvent–detergent (S/D) method was applied. Tri-*n*-butyl phosphate (solvent) and Triton X-100 (detergent) were removed from S/D treated immunoglobulins by ion-exchange chromatography on Q-Sepharose Fast Flow (FF). During the chromatographic procedure immunoglobulins remained bound on a Q-Sepharose FF, whereas solvent and detergent were eluted by washing with starting buffer. Elution of immunoglobulins was achieved by increasing the ionic strength of the starting buffer. The final immunoglobulin preparation contained less than 10 μg/ml of Triton X-100 and less than 2 μg/ml tri-*n*-butyl phosphate. It was confirmed that the S/D procedure did not cause a significant change in polymers and specific antibodies content. Immunoglobulin classes were also not affected by the same procedure. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Solvent–detergent method; Immunoglobulins; Triton X-100; Tributyl phosphate

1. Introduction

Beside the biological activity of the plasma derived products, the factor of outmost importance is their viral safety. Virus inactivation strategies are dictated by the lability of the virus and the stability of the biological activity of the protein in question. The solvent–detergent (S/D) method was generally accepted as an effective procedure for inactivation of enveloped viruses like human immunodeficiency virus (HIV), hepatitis B and C.

In the S/D method, Triton X-100 and tri-*n*-butyl phosphate (TnBP) are reagents commonly used for virus inactivation [1,2]. S/D reagents used in the process of virus inactivation have to be removed from therapeutic products. Therefore, to the presence of reagents for virus inactivation, as well as to their removal, special attention has to be given. By the clotting factors, which are plasma proteins, S/D reagents could be removed during the purification process by anion-exchange or affinity chromatography [3,4]. After S/D treatment of immunoglobulins, which are produced by cold ethanol fractionation, S/D reagents are usually removed using castor-oil and solid-phase extraction on reversed-phase support [5,6].

In this paper, we present the successful removal of Triton X-100 and TnBP from S/D treated immunoglobulins using ion-exchange chromatography on Q-Sepharose Fast Flow (FF).

2. Methods

2.1. Solvent/detergent treatment

Freeze-dried immunoglobulins (Institute of Immunology, Zagreb, Croatia, produced by the Kistler and

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Nitschmann procedure [7]), were dissolved in water for injection at pH 5.3 to 5.5 and filtrated through a 2-μm filter. Then, 0.3% (w/w) TnBP (Aldrich, Germany) and 1% (w/w) Triton X-100 (BDH, UK) were added and gently mixed for 6 h at room temperature.

2.2. Removal of S/D virus inactivation reagents

Removal of S/D reagents from immunoglobulins was performed by ion-exchange chromatography on Q-Sepharose FF (Pharmacia, Sweden). The gel was packed in a column (35×2.5 cm) and equilibrated in 0.05 *M* Tris, pH 8.3. The sample of S/D treated immunoglobulins was applied on the gel and the column was washed with ten column volumes of the starting buffer. The flow-rate was 24.4 ml/cm^2/h. Immunoglobulins were subsequently eluted with the same buffer containing 0.5 *M* sodium chloride. The elution was continued until the absorbance at 280 nm was less than 0.05. Elution of Q-Sepharose FF was continued with 2 *M* NaCl in the same buffer and with 0.5 *M* NaOH to remove pyrogenic substances. Other chromatographic parameters are given in the figure captions.

To determine the binding capacity of Q-Sepharose FF for immunoglobulins, several experiments were done. Different quantities (5, 10, 15 or 30 ml) of S/D treated immunoglobulins (136 mg/ml) were applied to the column which contained 170 ml of Q-Sepharose FF and subsequently eluted as already described. The protein content was determined in pool fractions eluted with 0.5 *M* NaCl in the starting buffer. The experimental conditions, under at least 95% of immunoglobulins bound to the matrix, were chosen for further experiments.

2.3. Ultra/diafiltration

The fractions obtained with 0.5 *M* NaCl in 0.05 *M* Tris, pH 8.3 were pooled and ultrafiltrated using Amicon stirred cells (YM 10 membrane). After the sample was diafiltrated, solid glycine was added to a final concentration of 0.3 *M* and the pH of the immunoglobulin solution was adjusted to 6.6.–6.7 and filtrated through a 0.22-μm membrane.

2.4. Analytical techniques

Protein concentration was determined by biuret method [8], using immunoglobulin as a standard. The remaining amount of TnBP was determined by gas chromatography, as described previously [9]. Residual Triton X-100 was determined by column switching HPLC method using analytical service from Bioanalitica (Zagreb, Croatia). Gel chromatography on Sephacryl S-300 HR was applied for quantitations 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 a radialimmunodiffusion technique using specific anti-human serum (Institute of Immunology, Zagreb, Croatia).

3. Results and discussion

Human immunoglobulins are plasma derivatives with a risk of transmitting viral infections. Despite the careful screening of individual plasma donors, the risk of transmission of some infectious diseases, like hepatitis (A, B, C) with intravenous immunoglobulins exists [10–12]. Usually, immunoglobulins are produced by ethanol fractionation methods [7,10] which were shown to be efficient for removal and/or inactivating HIV [13,14]. The method, effective in inactivating enveloped viruses like HIV, hepatitis B and C is known as the solvent–detergent method [1]. TnBP as a solvent and Triton X-100 or Tween 80 as a detergent were used. During the production of immunoglobulins, S/D reagents are usually removed in two steps: extraction with castor-oil and solid-phase extraction using a reversed-phase system [5,6].

In the present work immunoglobulins produced by cold ethanol fractionation according Kistler and Nitschmann [7] were treated with solvent (TnBP) and detergent (Triton X-100). After S/D treatment, reagents were removed using ion-exchange chromatography on Q-Sepharose FF. During the chromatographic procedure immunoglobulins were bound on

Table 1
Determination of protein content after application of different quantities of S/D treated immunoglobulins to the Q-Sepharose FF column (170 ml)

S/D treated immunoglobulins (proteins)			
Applied		Eluted	
ml	mg	mg	%
30	4080	2660	65.2
15	2040	1445	70.8
10	1360	1140	83.8
5	680	650	95.6

Q-Sepharose FF while S/D reagents were removed by washing the column with starting buffer. Chromatographic conditions, especially ionic strength of the equilibration buffer and sample load size were carefully chosen to prevent binding of S/D reagents and in that way increase the capacity of the gel for immunoglobulins. Determination of the Q-Sepharose FF gel capacity for immunoglobulin binding is shown in the Table 1. Conditions where capacity was approximately 4 mg of immunoglobulins per 1 ml gel gave the best recovery of immunoglobulins (>95%), although the capacity of Q-Sepharose FF for immunoglobulins was much higher (6–15 mg/ml gel). Therefore, 5 ml (680 mg of proteins) of S/D treated immunoglobulins was applied to 170 ml of Q-Sepharose FF. Most of the S/D reagents flowed through the column (Fig. 1, peak 1). It was confirmed that for efficient removal of S/D reagents the gel had to be washed with ten column volumes of the starting buffer. Immunoglobulins were eluted with 0.5 *M* NaCl in the same buffer (Fig. 1, peak 2), and residual proteins were eluted with 2 *M* NaCl (Fig. 1, peak 3). S/D reagents and proteins were determined in the different pool-fractions. The first peak contains about 90% of S/D reagents and 3–4% of immunoglobulins, which were eluted with three column volumes. The rest of the S/D reagents were removed using additional six to eight column volumes of the starting buffer (Table 2). Most of the immunoglobulins (>95%) were collected in fractions 201–220 (Fig. 1, Table 2) and this pool contained traces

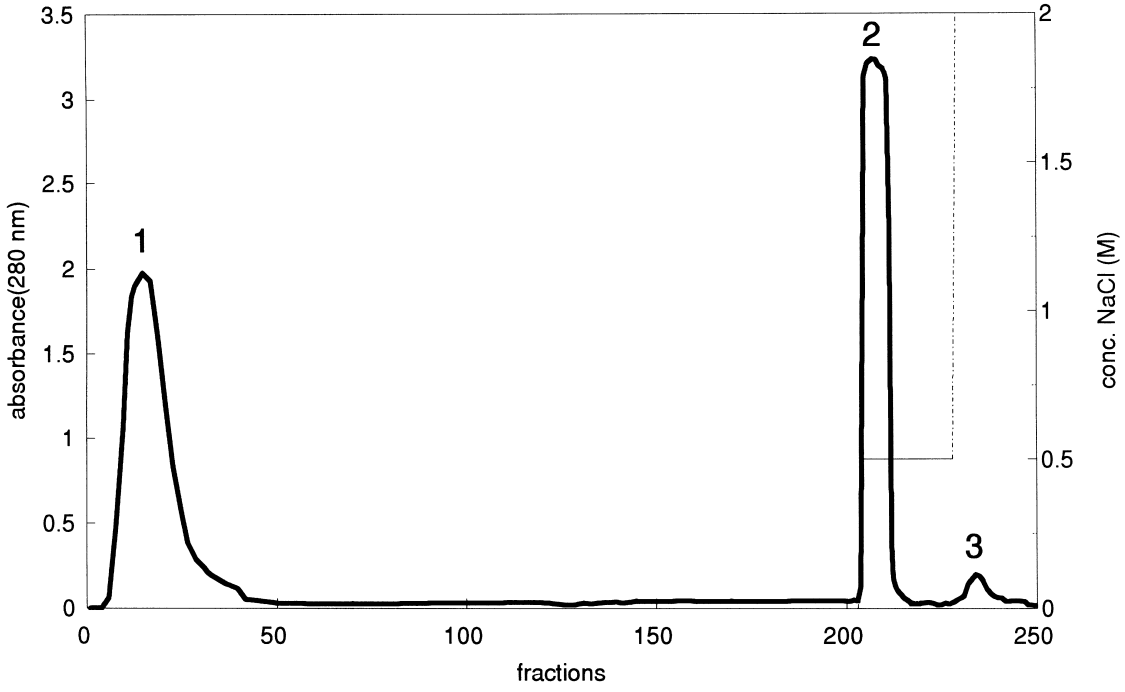

Fig. 1. Ion-exchange chromatography of S/D treated immunoglobulins on Q-Sepharose FF. Column: 35×2.5 cm. Sample: 5 ml (680 mg of proteins). Eluents: 0.05 *M* Tris pH 8.3 (starting buffer), 0.5 *M* NaCl in 0.05 *M* Tris pH 8.3, 2.0 *M* NaCl in 0.05 *M* Tris pH 8.3. Fractions: 10 ml.

Table 2
Determination of immunoglobulin, TnBP and Triton X-100 in fraction-pools after chromatography on Q-Sepharose FF (Fig. 1)[a]

Fraction-pools		Proteins		TnBP		Triton X-100	
Fraction (number)	Volume (ml)	Eluted (mg)	Recovery (%)	Eluted (mg)	Recovery (%)	Eluted (mg)	Recovery (%)
0–50	−500	20	2.9	13.6	90.7	45.0	90.0
51–100	−500	5	0.7	0.9	6.0	3.5	7.0
101–200	−1000	nd[b]	–	nd	–	nd	–
201–220	−200	650	95.6	0.15	1.0	0.7	1.4
221–250	−300	7	1.0	nd	–	nd	–
250	−2500	682	100.2	14.65	97.7	49.2	98.4

[a] Note: 5 ml of S/D-treated immunoglobulins applied on Q-Sepharose FF contain 680 mg of immunoglobulin (100%), 15 mg TnBP (100%), 50 mg Triton X-100 (100%).
[b] nd: not detected.

of S/D reagents (Table 2). It was shown that during the chromatography about 5% of immunoglobulins were lost. After chromatography the fractions from peak 2 were pooled and ultra/diafiltrated using Amicon ultrafiltration stirred cells.

The immunoglobulins were formulated (glycine added, pH adjusted and filtrated through a 0.22-μm membrane) and submitted for further analytical testing. During the S/D treatment removal of S/D reagents and formulation of immunoglobulins, about 20% of immunoglobulins were lost. The final immunoglobulin preparations contain less than 10 μg/ml of Triton X-100 and less than 2 μg/ml of TnBP. No significant changes in polymer content, the content of specific antibodies or classes of immunoglobulin were observed.

It was shown that the chromatographic method is probably an efficient way to eliminate TnBP and Tween 80 or Triton X-100 during the manufacturing process of clotting factors. Burnouf et al. [3] treated F VIII:c concentrate with 0.3% TnBP and 1.0% Tween 80 to inactivate lipid-enveloped viruses. Most TnBP–Tween 80 flowed through the DEAE-Fractogel TSK 650 unretarded. They determined the low level of TnBP (4.1 μg/ml) and Tween 80 (<10 μg/ml) in the final therapeutic product.

Heparin-Sepharose FF [4] could also be used for the same purpose.

In this paper we present a new possibility for removal of solvent and detergent from S/D treated immunoglobulins using ion-exchange chromatography on Q-Sepharose FF.

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