Journal of Chromatography, 201 (1980) 281–286
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,200

APPLICATION OF CM-SEPHADEX C-50 FOR THE PRODUCTION OF IMMUNOGLOBULINS

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SUMMARY

A combined precipitation–ion-exchange chromatographic method is described for the production of immunoglobulin G (IgG) from human plasma. The crude IgG fraction was precipitated with polyethylene glycol (PEG) and further purified on CM-Sephadex C-50. PEG and the contaminating proteins were not bound to the ion-exchange resin; they were eluted with the starting buffer. The purified IgG fraction was obtained in the second step by elution of the proteins bound to the column. In addition to column chromatography a batch procedure has been developed that requires relatively simple equipment. IgG of high purity was obtained by both column chromatography and batch procedure.

INTRODUCTION

The predominantly used method for large-scale processing of protein preparations for therapeutic purposes is the Cohn cold ethanol procedure[1]. A great disadvantage and the main difficulty of this technique is that cooling is a basic requirement. Therefore many new methods have been suggested as alternatives.

Polson *et al.*[2] have described the fractionation of plasma proteins, the processing of immunoglobulins and fibrinogen with polyethylene glycol (PEG). In this method ammoniumsulphate, ethanol and ion-exchange chromatography are used for removing the remaining traces of PEG from the immunoglobulin fraction[3]. Curling *et al.*[4] elaborated on a chromatographic method for the purification of the albumin fraction precipitated by PEG. Albumin is bound to DEAE-Sepharose; PEG and most of the other contaminating proteins are eluted from the column with the starting buffer solution. The purpose of our work was to purify the crude immunoglobulin G (IgG) fraction precipitated by PEG with ion-exchange chromatography. We have developed a batch procedure which can be carried out with relatively simple equipment.

EXPERIMENTAL

Fractionation of human plasma with PEG

Human plasma was fractionated with PEG 4000 (Hoechst, Frankfurt/M,

0021-9673/80/0000–0000/$02.25

G.F.R.) according to the method described by Haskó[5]. The first IgG fraction was precipitated at pH 6.5 by 15% w/v PEG at room temperature, hereafter the precipitate was centrifuged. The albumin fraction was obtained from the supernatant by further addition of PEG. The crude IgG fraction was dissolved in distilled water at pH 8.0 and thus the solution was brought to a 6–7% concentration of protein. Hereafter, the solution still contains about 4% PEG, which precipitates the fibrinogen after standing for one night at 4°C. The pH was then adjusted to pH 6.7 with HCl and the precipitate was removed by centrifugation.

Ion-exchange chromatography

Chromatography of the crude IgG fraction was performed on a CM-Sephadex C-50 column (Pharmacia, Uppsala, Sweden).

Gel was swollen in distilled water and then equilibrated with 0.01 *M* (pH 6.7) sodium citrate–sodium chloride buffer. The bed volume was 40 cm^3 gel and the run volume of the sample was 20 cm^3 crude IgG fraction (1.2–1.4 g protein/g dry Sephadex).

Contaminating proteins and PEG were eluted from the column with 2 volumes of the starting buffer. IgG bound to the column was eluted with 0.01 *M* sodium citrate containing 0.25 *M* sodium chloride (pH 7.8). The fractions were collected by an Ultrorac 7000 (LKB, Stockholm, Sweden) collector. Proteins separated by chromatography were detected on a LKB Uvicord II photometer at 280 nm.

Batch procedure

CM-Sephadex gel was equilibrated as above with 0.01 *M* sodium citrate–sodium chloride buffer (pH 6.7). Hereafter, the excess of the buffer was drawn off on a Buchner funnel. 1000 cm^3 crude IgG solution was mixed to 2400 cm^3 gel prepared in this way. The gel was then left standing for half an hour. Afterwards the non-adsorbed proteins and PEG were drawn off and the gel was washed with three volumes of the starting buffer. Excess buffer was again removed and 500 cm^3 0.01 *M* sodium citrate buffer (pH 7.8) containing 0.25 *M* sodium chloride was added. The mixture was stirred for 30 min. The eluted IgG fraction was drawn off on a Buchner funnel and to reduce the loss of IgG to the minimum the gel was washed twice with 250 cm^3 of the eluting buffer. The obtained solutions were mixed, resulting in the purified IgG fraction.

Concentration of protein solutions

IgG solutions obtained either by means of chromatography or by the batch procedure were concentrated to 6 and 16.5% by ultrafiltration on a Bio-Fiber 80 hollow fiber ultrafilter (Bio-Rad Labs., Richmond, CA, U.S.A.) or by negative pressure dialysis.

Analytical methods

The protein content of the samples were assayed by the biuret method. The protein composition of the samples were determined by electrophoresis densitometry on cellulose acetate membrane (Gelman, Ann Arbor, MI, U.S.A.). Purity of the fractions was determined by Scheidegger[6] immunoelectrophoresis, polyvalent anti-human horse serum (Human, Gödöllö-Budapest, Hungary) was used as antibody.

PEG remaining in the protein solutions was detected with Nessler-Winkler reagent[2]. Biological tests such as sterility, pyrogenicity and toxicity were made according to *Pharmacopoea Hungarica*, Ed. VI.

RESULTS

Practically all IgG was precipitated from the plasma with 15% PEG at pH 6.5. After dissolution of the precipitate the protein concentration was 6–7%, containing 45–50% IgG. After precipitation of fibrinogen the supernatant contained about 90% of the plasma IgG.

During ion-exchange chromatography two protein fractions were obtained. Fig. 1 shows the elution profile by column chromatography. The first peak contains albumin, α- and β-globulins. PEG was eluted together with the globulins. IgG was eluted from the column by 0.25 *M* sodium chloride in 0.01 *M* (pH 7.8) sodium citrate buffer. Fig. 2. shows the immunoelectrophoretogram of the single fractions. The protein concentration of the eluted (*ca.* 97% pure) IgG fraction was in the range 1.8–2.4% depending on the initial IgG content of the sample. As only 3–7% of the initial IgG content is desorbed by the starting buffer, the IgG yield may reach 95%. Table I displays the average values of IgG purity and yield.

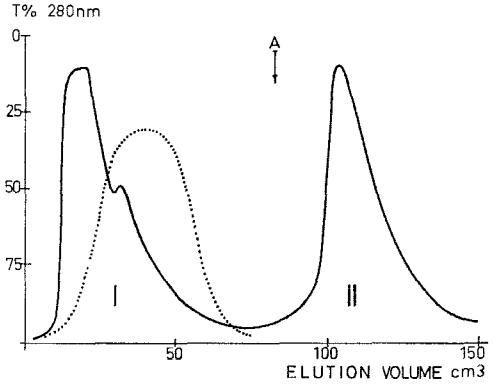

Fig. 1. Chromatogram of crude IgG fraction precipitated by PEG on a CM-Sephadex C-50 column. Column: Pharmacia K 15/30. Bed volume: 40 cm³, sample volume: 20 cm³, starting buffer: 0.01 *M* (pH 6.7) sodium citrate–sodium chloride. A = 0.01 *M* (pH 7.8) sodium citrate + 0.25 *M* sodium chloride. ..., PEG. I = Non-adsorbed proteins; II = IgG fraction.

TABLE I

COMPARISON OF THE AVERAGE YIELD AND PURITY OF IgG PREPARATIONS OBTAINED BY COLUMN CHROMATOGRAPHY AND BATCH PROCEDURE FROM CRUDE IgG FRACTIONS

Method	*IgG content of the fractions*		*Yield (%)*	*Purity (%)*
	Crude IgG (g)	*Purified IgG (g)*		
Column chromatography $n = 20$	0.65 ± 0.10	0.62 ± 0.09	95 ± 2	97 ± 1
Batch procedure $n = 10$	27.00 ± 4.04	24.84 ± 3.70	93 ± 2	95 ± 1

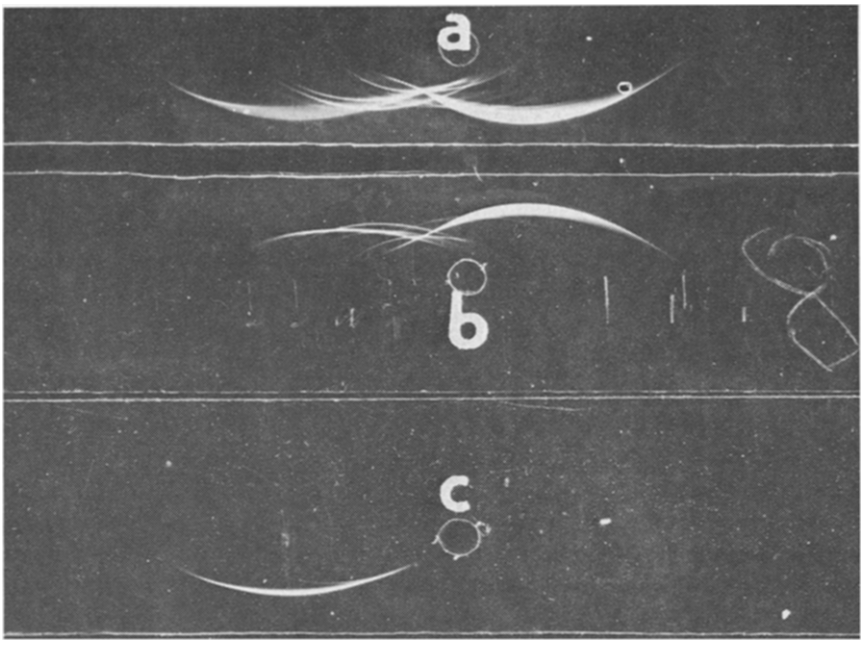

Fig. 2. Immunoelectrophoretogram of fractions obtained by CM-Sephadex column chromatography. a = Crude IgG fraction, b = non-adsorbed proteins, c = purified IgG, antibody = antihuman polyvalent horse serum.

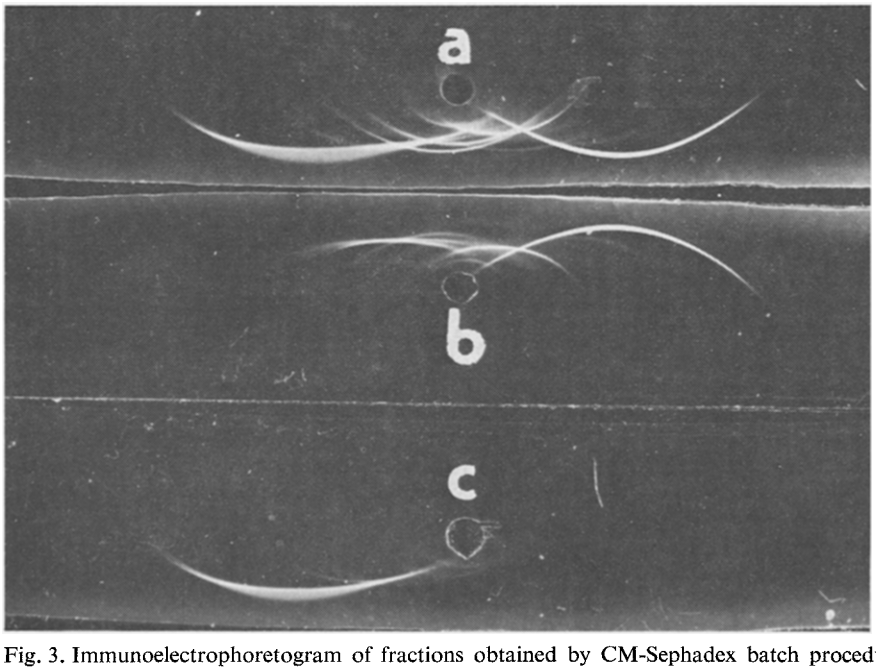

Fig. 3. Immunoelectrophoretogram of fractions obtained by CM-Sephadex batch procedure. a = Crude IgG fraction, b = non-adsorbed proteins, c = purified IgG, antibody = antihuman polyvalent horse serum.

The technically more simple batch procedure is also suitable for purifying the crude IgG fraction on CM-Sephadex. In our experiments we used 4 l of human plasma as starting material. 0.01 *M* (pH 6.7) sodium citrate–sodium chloride buffer removed all of the PEG and contaminating proteins. The protein concentration of the purified IgG eluted from the gel by 0.25 *M* sodium chloride in 0.01 *M* (pH 7.8) sodium citrate was in the range 1.2–1.6%. The immunoelectrophoretogram of protein fractions obtained by batch procedure is shown in Fig. 3. This method results in an average yield of 93% and 95% purity. Table I displays the average values of purity and yield in column chromatography and batch procedure. The purified IgG fractions were concentrated to 16.5% by ultrafiltration. The purification of IgG with the batch method was made under sterile conditions. The products controlled by the biological tests proved to be sterile, pyrogen-free and nontoxic.

DISCUSSION

Our purpose was to obtain a high yield of pure IgG from the crude IgG fraction obtained by PEG fractionation. CM-Sephadex was used for binding IgG, as this proved to be the most simple mode for purifying the crude IgG fraction and for removing the traces of PEG. In the first step of our procedure, PEG and the contaminating proteins were eluted, in the second step IgG was eluted. This technique proved suitable for practical purposes. High purity IgG was obtained with both column chromatography and batch procedure. Although the mean value of purity of the IgG fraction obtained with the batch procedure is somewhat (2%) lower than column chromatography as displayed in Table I the products still meet the purity requirements of the WHO[8]. Thus, this is a three-step method resulting in the production of purified product suitable for therapeutic purposes. The first step is the precipitation of IgG with PEG, the second step is the purification of the precipitated IgG with ion-exchange chromatography and the third step is the concentration and sterile filtration of the product. In comparison to the cold ethanol method of Cohn[1] or Nitschmann[7], where several precipitation steps, filtration and removal of ethanol are necessary, our technique saves a considerable amount of work.

This technique shows several advantages over those reported in the literature, which apply DEAE-ion-exchange resins. DEAE-ion-exchangers do not bind IgG, thus PEG cannot be removed from the product. PEG has no electric charge and is not bound by the ion-exchange resin. Our batch procedure renders the possibility of obtaining greater amounts of IgG for therapeutic purposes, without investment of expensive apparatus. This technique is especially economic if smaller amounts of plasmas containing specific immunoglobulins (for example anti-hepatitis, anti-varicella) are used. The clinical applicability of this product is supported by biological tests such as sterility, pyrogenicity and toxicity. Our further aim is to develop this technique for the production of intravenous IgG.

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