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Journal of Chromatography A, 766 (1997) 49–60

JOURNAL OF CHROMATOGRAPHY A

Screening of a large number of dyes for the separation of human immunoglobulin G2 from the other immunoglobulin G subclasses Immunoglobulin G2 enrichment on immobilized Procion Yellow HE-4R

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Received 30 July 1996; revised 26 November 1996; accepted 28 November 1996

Abstract

Human immunoglobulins (IgG) are produced on a multi-ton scale for therapeutic applications. There is presently no available method to manufacture IgG preparations enriched with immunoglobulins from the IgG2 subclass although they might be useful for therapeutic purposes. By frontal chromatography, we have screened 69 immobilized dyes, among which, six display a different affinity for IgG2 and other subclasses. One (Procion Yellow HE-4R) was studied further. The screening of various mobile phase conditions allowed us to devise a procedure to prepare IgG2 enriched IgG solutions: The cumulative yield for IgG2 was 43% and IgG2/total IgG ratio in the final product was 67%.

Keywords: Preparative chromatography; Immobilized dyes; Affinity ligands; Stationary phases, LC; Dyes; Immunoglobulins; Proteins

1. Introduction

IgG are important therapeutic agents and every year some 27 tons are administered to patients [1].

IgG are classified as IgG1, IgG2, IgG3 and IgG4. Structures of the constant regions of all subclasses have been characterized [2].

One of the indications of IgG administration is to enhance resistance of the patient to infections. In some cases, a specific pathogen is identified and specific immunoglobulins are prescribed to treat or to prevent the outbreak of the disease (e.g., tetanus). These specific immunoglobulins are prepared on an industrial scale by affinity chromatography using immobilized specific ligands for the antibodies [3]. In other cases, no definite pathogen is identified but IgG infusion is prescribed to enhance the patient's resistance to bacterial pathogens. Most often, antibodies directed against bacterial pathogens belong to the IgG2 subclass [4]. Hence, in situations of bacterial infections, it would be advantageous to use IgG2-enriched IgG preparations.

Moreover, IgG2 congenital deficiencies are characterized by a high susceptibility to bacterial infections. These patients are presently treated by total

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PII S0021-9673(96)01018-7

IgG transfusions [5]. A more logical treatment would be an infusion of IgG2-enriched IgG preparations.

At present, there is no established method for the preparation of IgG2-enriched solutions suitable for therapeutic use. Chromatography on anion exchangers allows the preparation of pure IgG4 [6] but no enrichment of IgG2 was obtained.

Some separation of IgG2 from other IgG subclasses is possible on immobilized protein A. When a protein A–Sepharose column loaded with human serum was developed with a decreasing pH gradient [7], two fused optical density peaks were obtained (the first was enriched with IgG2 and the second, which was separated from the former by 0.4 pH units, contained a low amount of IgG2). A second pass on the column allowed fractions with IgG2 with a very high purity level (of 95%) to be obtained, but quantitative data on IgG2 yield were lacking. Similar experiments have been performed using Protein A–Superose, again, fractions with high purity levels of IgG2 were obtained but data on the yield were not given [8].

While Protein A has no significant affinity for IgG3, protein G (which is another bacterial receptor for immunoglobulins, isolated from Streptococci) was found to retain all human IgG subclasses [9]. To date, to the best of our knowledge, no systematic study of immobilized protein G as a tool to separate IgG subclasses has been performed.

In a careful study of the operating conditions for chromatography of IgG on T gel and a variety of hydrophobic interaction chromatography supports, Bridonneau and Lederer [10] demonstrated that only T gel could display some selectivity for human IgG subclasses: IgG was loaded on a T gel column equilibrated in a mobile phase without added salt, the non-retained peak contained 50% of the loaded IgG2 (which represented 50% of the total IgG in this peak, compared to 35% in the loaded material).

Histidine immobilized on agarose was proved to be an interesting ligand for the separation of human IgG subclasses [11]. The development of a column with an ionic strength step gradient allowed the authors to obtain a fraction containing pure IgG2 (as assessed through immunoelectrophoresis), but with a rather low yield (13%) of IgG2 (the remaining part of IgG2 was recovered in unretained fractions that were largely contaminated with other IgG subclasses). Histidine was also immobilized on mechanically resistant supports, but no data were given on the separation of IgG subclasses on these supports [12].

Birkenmeier and Dietze [13] described the separation of plasma proteins on a membrane derivatized with a ligand of undisclosed structure. When human serum was loaded onto the affinity membrane, only immunoglobulins were retained and these were eluted using increasing ionic strength. This affinity membrane showed some specificity for IgG subclasses, since, if pure immunoglobulins were loaded onto the membrane, 72% of the loaded IgG2 was recovered in the non-retained fraction; the proportion of IgG2 in the excluded peak was 41% of the total IgG versus 25% in the starting material [13].

In this context, we decided to assess if chromatography on immobilized dyes could provide a suitable method to prepare IgG2-enriched IgG solutions. Immobilized dye is the most used affinity chromatography ligand for the large scale preparation of proteins [14]. Immobilized Cibacron Blue F3GA has been used for large-scale preparation of human albumin for therapeutic use [15,16]. Protocols for laboratory-scale isolation of murine immunoglobulins using this dye have been described (e.g., Ref. [17]). The capacities of many dyes for human and animal immunoglobulins have been evaluated by screening, performed by centrifugal chromatography [18]. Since it is well established that some immobilized dyes have a very high selectivity that allows the separation of closely related proteins (for example, isozymes of lactate dehydrogenase [19]), it was tempting to check if dyes could be used to separate IgG2 from other IgG subclasses.

In fact, some 25 years ago, Byfield et al. [20] demonstrated that fractionation of immunoglobulins was possible using immobilized dye chromatography. When IgG was loaded onto immobilized Remazol Yellow GGL that had been equilibrated in 20 m*M* sodium phosphate buffer, pH 7.4, approximately half of the IgG was retained by this immobilized dye and the other half was eluted from the column using 1 *M* NaCl in the same buffer. However, IgG1 and IgG2 were approximately equally distributed in the excluded and eluted peaks (by contrast, IgG4 was not retained by the gel, while most of the IgG3 was retained and eluted using 1 *M* NaCl).

In order to devise an efficient method to separate IgG2 from total IgG on a preparative scale by immobilized dye chromatography, we decided to screen a large number of immobilized dyes. We tried to identify dyes that had a large capacity for immunoglobulins and also had different affinities for IgG2 and the other IgG subclasses. Hence, we decided to use frontal chromatography, which allows the evaluation of both capacities and relative affinities [21], instead of zonal chromatography, as formerly used by others [18].

2. Experimental

2.1. Materials

Dyes were donated by the following dye manufacturers: Sandoz Chimie (presently known as Clariant, Rueil Malmaison, France), ICI France (presently known as Zeneca Colours, Lyon, France) and Hoechst France (Puteaux, France). A list of dyes used in this study is provided in Table 1, together with the abbreviations used to identify them later in the text. Sepharose CL 4B and Ultrogel A4, which were used for dye immobilisation, were from Pharmacia (Uppsala, Sweden) and Biosepra (Gennevilliers, France), respectively. Chemicals were bought from Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) or Bio-Rad (Richmond, CA, USA). Purified immunoglobulins were from Biotransfusion (Les Ulis, France)

2.2. Dye immobilization

The immobilization procedure (derived from published procedures, [22]) was performed as follows; Sepharose CL 4B was rinsed extensively with water and then passed through 0.2 *M* NaOH containing 2% (w/v) NaCl. The gel was transferred to a vessel containing reactive dye (20 mg per millilitre gel; the dye was used as obtained from the manufacturer). Then, a solution containing 0.2 *M* NaOH and 2% NaCl was added so that the final reaction volume was twice the gel volume. The gel suspension was rotated at 60°C for 1 h, then rinsed with 10 volumes of 0.2 *M* NaOH and with 50% dimethyl sulfoxide in water until clear washings were obtained. It was finally rinsed with water and stored as a suspension in 100 m*M* NaCl containing 0.02% sodium azide. In order to obtain gels with a higher dye substitution level, the coupling procedure was repeated three times (gels are noted by adding "(3)" to the dye's abbreviation). The dye substitution was measured as follows [22]: The immobilized dye was hydrolyzed in 50% acetic acid at 110°C until complete dissolution of the gel occurred. The dye substitution level was then evaluated by spectrophotometry. The wavelength for measurement was chosen in the visible range at the absorbance peak of the dye. The substitution levels are expressed as mg dye/ml of support, using a reactive dye (as obtained from the manufacturer) as the standard.

2.3. Preparation and characterization of immunoglobulins used as starting material for the chromatographic procedures

We used immunoglobulins prepared from human plasma by the Kistler and Nitschmann procedure [23]. These immunoglobulins were manufactured for administration by an intravenous route, thus, they were treated with dilute pepsin [24]. They were characterized by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) run under both reducing and non-reducing conditions [25]. IgG subclasses were assayed by enzyme-linked immunosorbent assay (ELISA) using a previously described procedure [26].

Prior to chromatography, immunoglobulins were passed through a Sephadex G-25 column that had been equilibrated in buffer A (10 m*M* KOH, adjusted to pH 6.0 with solid 2-(N-morpholino)ethanesulfonic acid, MES).

2.4. Frontal chromatography

Frontal chromatography was performed using 1.3 cm high columns (1 cm I.D.). Immobilized dye columns were equilibrated with buffer A before loading. The flow-rate was 3 ml/h and 0.5 ml fractions were collected. Four chromatographic procedures were routinely performed in parallel using a multichannel peristaltic pump and a laboratory-modified four-channel collector.

After completion of the frontal chromatography,

Table 1
Names of dyes used in this study

Abbreviation	Dye	Manufacturer
R_1	Procion Red HE-3B	Zeneca Colours
R_2	Procion Red MX-8B	Zeneca Colours
R_3	Procion Red HE-7B	Zeneca Colours
R_5	Procion Red P-8B	Zeneca Colours
R_6	Procion Red MX-7B	Zeneca Colours
R_7	Procion Red P-4BN	Zeneca Colours
R_8	Procion Red MXG	Zeneca Colours
R_9	Procion Red MX-5B	Zeneca Colours
R_{11}	Procion Scarlet P-2R	Zeneca Colours
R_{12}	Procion Rubine MXB	Zeneca Colours
R_{13}	Remazol Brilliant Red 6B	Hoechst
R_{15}	Remazol Brilliant Red BB	Hoechst
R_{17}	Remazol Brilliant Red RB	Hoechst
R_{19}	Drimarene Brilliant Red R-4BL.CDG	Clariant
O_1	Procion Orange MX-2R	Zeneca Colours
O_2	Procion Orange P-2R	Zeneca Colours
O_3	Procion Orange HER	Zeneca Colours
O_4	Procion Orange MX-G	Zeneca Colours
O_5	Remazol Brilliant Orange 3R	Hoechst
O_6	Drimarene Brilliant Orange R-R	Clariant
Y_1	Procion Yellow P-3R	Zeneca Colours
Y_2	Procion Yellow MX-4R	Zeneca Colours
Y_3	Procion Yellow P4G	Zeneca Colours
Y_4	Procion Yellow MX-4G	Zeneca Colours
Y_6	Procion Yellow MX-8G	Zeneca Colours
Y_7	Procion Yellow HE-6R	Zeneca Colours
Y_8	Procion Yellow HE-4R	Zeneca Colours
Y_9	Procion Yellow HE-6G	Zeneca Colours
Y_{11}	Procion Yellow SP-8G	Zeneca Colours
Y_{12}	Procion Yellow HE-3G	Zeneca Colours
Y_{13}	Procion Yellow MX-GR	Zeneca Colours
Y_{14}	Procion Yellow GR	Zeneca Colours
Y_{15}	Remazol Yellow GL	Hoechst
Y_{16}	Remazol Yellow 4GL	Hoechst
Y_{17}	Remazol Yellow RNL	Hoechst
G_1	Procion Green HE-4BD	Zeneca Colours
G_2	Procion Green P4-BD	Zeneca Colours
G_3	Procion Olive Green P-7G	Zeneca Colours
G_4	Remazol Brilliant Green 6B	Hoechst
T_1	Procion Turquoise H-A	Zeneca Colours
T_2	Procion Turquoise SP-2G	Zeneca Colours
T_3	Procion Turquoise MX-G	Zeneca Colours
T_4	Procion Turquoise PC-X	Zeneca Colours
B_4	Procion Navy HER-150	Zeneca Colours
B_6	Procion Blue SP-3R	Zeneca Colours
B_7	Procion Blue MX-G	Zeneca Colours
B_9	Procion Blue MX-2GN	Zeneca Colours

Table 1. Continued

Abbreviation	Dye	Manufacturer
B_{10}	Procion Blue HE-RD	Zeneca Colours
B_{11}	Procion Blue MX-7RX	Zeneca Colours
B_{14}	Procion Blue P-5R	Zeneca Colours
B_{15}	Procion Blue MX-4GD	Zeneca Colours
B_{16}	Procion Blue HE-GN	Zeneca Colours
B_{17}	Procion Blue P-7RX	Zeneca Colours
B_{18}	Procion Navy HE-RN	Zeneca Colours
B_{21}	Drimarene Blue R-3GL	Clariant
B_{22}	Drimarene Navy Blue R-BNN	Clariant
V_1	Procion Purple P-3R	Zeneca Colours
V_2	Drimarene purple R-2RL.CDG	Clariant
A_1	Procion Black P-2R	Zeneca Colours
A_2	Procion Black HEXL	Zeneca Colours
A_3	Procion Black PN	Zeneca Colours
A_4	Remazol Black B	Hoechst
A_6	Drimarene Black R-3B	Clariant
H_1	Diazol Light Grey 6BLN	Zeneca Colours
C_1	Procion Brown MX-GRN	Zeneca Colours
C_3	Procion Brown H-3R	Zeneca Colours
C_4	Procion Brown P-GR	Zeneca Colours
C_5	Procion Brown HE-XL	Zeneca Colours
C_6	Procion Brown P-2R	Zeneca Colours
C_7	Procion Brown MX-5BR	Zeneca Colours

Manufacturers and abbreviations used elsewhere in the text to identify the dyes are also indicated.

the columns were rinsed with buffer C (buffer A containing 2 *M* NaCl and 6 *M* urea) and finally with 100 m*M* NaCl containing 0.02% sodium azide for storage.

2.5. Analysis of chromatographic fractions

Each fraction was assayed for protein content by the Bradford assay [27], which was adapted for use in microtitre plates. Fractions selected after the Bradford assay were analyzed for IgG2 content and for total IgG by ELISA [26].

2.6. Screening of several eluents in order to devise mild procedures for elution from immobilized Procion Yellow HE-4R

Immobilized dye was sedimented to a height of 2.6 cm in a 1 cm diameter column. It was then equilibrated in buffer A. The column was operated at a flow-rate of 20 ml/h. An 8-mg amount of purified IgG was loaded and the column was rinsed with 5 ml of buffer A. It was then developed with 20 ml of a solution of the tested eluent dissolved in buffer A at various concentrations (see Section 3). The solutes tested as potential eluents were AMP and ATP (sodium salts), inositol hexaphosphate (dodecasodium salt) and NaH_2PO_4. The pH value was checked and readjusted to pH 6.0 with dilute HCl or dilute NaOH, as needed. Thereafter, the column was developed with buffer B (buffer A containing 2 *M* NaCl) until the recorder trace returned to the baseline level, and finally, with buffer C. Fractions collected during development of the column with the tested eluent and with buffer B were separately pooled and assayed for IgG2 and total IgG by ELISA.

We also investigated if raising the pH could be

used to elute IgG2 with some selectivity: The sample was loaded on the column (which was equilibrated in buffer A). Thereafter, the column was developed with 20 ml of 10 m*M* KOH containing 30 m*M* NaCl, adjusted to the desired pH value using MES (for pH values between 5.5 and 6.5) or with N-(2-hydroxyethyl)piperazine-N′-2-ethane sulfonic acid (HEPES, for pH values between 6.5 and 8.5). Thereafter, the column was developed with the same buffer containing 2 *M* NaCl (called buffer B′), and finally with buffer C (containing urea). Inositol hexaphosphate (IHP), dissolved at various concentrations in mobile phase, pH 7.5, was also tested as an eluent (see Section 3).

For each tested mobile phase condition it was checked, by assaying IgG2 and total IgG in pooled fractions corresponding to buffer B or B′ and to buffer C, that a quantitative yield for immunoglobulins was obtained. The amount of IgG present in fractions eluted with buffer C was in every case lower than 10% of the IgG loaded.

2.7. IgG2 enrichment of igG solutions on immobilized Procion Yellow HE-4R

A preparative procedure to produce IgG2-enriched IgG solutions was devised. Gel that had been coupled three times was used. Two passes on the same Y8 column were performed: IgG2 elution was obtained by increasing the pH of the mobile phase on the first pass, and using an IHP concentration gradient for the second pass, as described below: An 8-mg amount of total IgG was loaded on a 1-cm I.D. 2.5 cm high column packed with immobilized Y8 that had been equilibrated in buffer A (20 ml/h flow-rate). After loading, the column was rinsed with three column volumes of buffer A. The column was then developed using a buffer, pH 8, that was prepared as mentioned above. The fractions eluted with buffer at pH 8 were then simply readjusted at pH 6.0 with dilute HCl and again loaded on the same column, which had been re-equilibrated in buffer A. Finally, the column was developed with an IHP concentration gradient from 0 to 50 m*M*. The ELISA assay (Fig. 5) allowed fractions to be pooled so that an IgG solution complying with preset specifications could be obtained.

3. Results and discussion

3.1. Characterisation of the IgG preparation used as the starting material for the chromatographic procedure

The IgG used as the starting material showed a band pattern that was characteristic of intact IgG (not shown) on SDS–PAGE run under reduced and unreduced conditions, even though this material (intended for intravenous use) had been treated with dilute pepsin to remove IgG aggregates [24].

The proportions of IgG subclasses present in the starting material were 67% for IgG1, 28% for IgG2 and 6% for IgG4. No IgG3 was present, reflecting the known sensitivity of this IgG subclass to proteases and is evidence of the pepsin treatment.

3.2. Capacities of the immobilized dyes for total IgG

Results are shown in Fig. 1 which ranks the various immobilized dyes as a function of their capacity for IgG. The *y*-axis indicates the volume eluted from the column at which the concentration of IgG in the collected fractions was 50% of the IgG concentration in the incoming solution (50% breakthrough volume). This value of 50% is arbitrary, hence, the precise ranking would have been different if another limiting value had been chosen, since the breakthrough curve was not as steep with all dyes. It is obvious, as noted before [21], that capacities of dyes may depend on the actual amount of each dye immobilized on agarose. For example R6 and Y8 have been immobilized as described in Section 2 at two different concentrations: R6 and Y8 contained 1.8 and 1.3 mg dye/ml gel, respectively, and their 50% breakthrough volumes were 25 and 46 ml, respectively. R6(3) and Y8(3) were both substituted at 3.0 mg dye/ml gel and their capacities for total IgG were significantly increased (breakthrough volumes were 35 and 60 ml, respectively).

Increasing dye substitution does not always lead to increased capacity, as observed with R17. R17 substitution and breakthrough values were 1.7 mg/ml and 26 ml while the figures for R17(3) were 2.54 mg dye/ml gel and only 28 ml. In the careful studies of Wirth et al. [28] on the influence of the dye

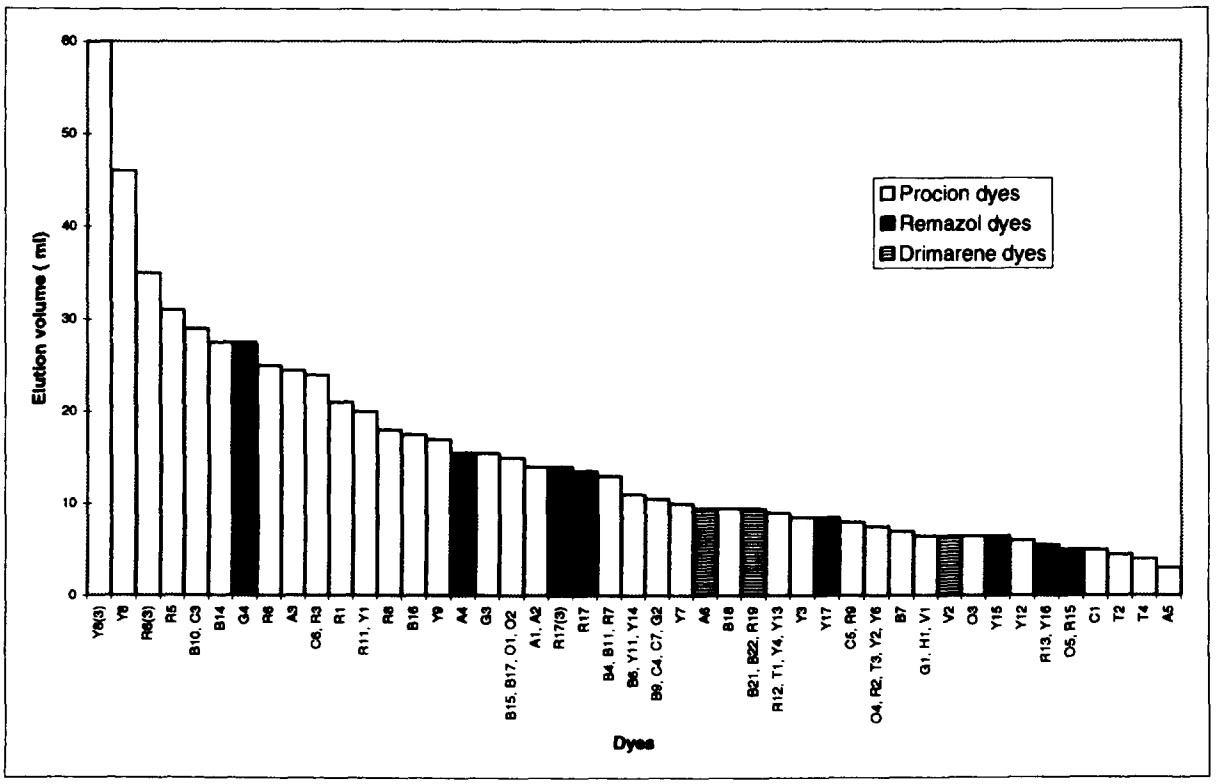

Fig. 1. Capacities of the immobilized dyes used in this study for IgG. Capacities were measured as described in Section 2 by frontal chromatography of IgG solutions (0.63 mg/ml) on 1 ml columns. The *y*-axis indicates the volume eluted from the column at which the concentration of IgG in the collected fractions was 50% of the IgG concentration in the incoming solution. Experimental values were reduced by the 50% elution volume of an unsubstituted agarose column. "(3)" following dye abbreviations indicates that the immobilized dye has been prepared using three coupling cycles (see Section 2).

substitution level on the properties of immobilized dyed sorbents, it was shown that increased substitution could lead to reduced capacity. This was attributed by the authors to the onset of some steric hindrance, impeding protein dye interaction in heavily substituted gels.

Noticeably, we did not observe that selectivity of the immobilized dyes for the IgG subclasses was influenced by the substitution level (see below).

3.3. Correlations between dye structures and capacities for IgG

If most of the structures of the chromophores of the immobilized dyes are undisclosed, structures of the reactive groups are known. Remazol dyes are anchored to the fabrics by a vinyl sulfonic group obtained by hydrolysis of the hydroxyethylsulfonic group during the dying process [29] (Fig. 2). This vinyl sulfonic group is reminiscent of the divinyl sulfone activation of agarose, which is part of the synthesis of T gels [30]. Nevertheless, Remazol dyes did not show a significantly higher capacity for IgG than Procion dyes, which are anchored to the gel by a triazinic group (see Fig. 1). Drimarene has a monochloro difluoro pyrimidimium ring for attachment to fabrics (or agarose) [29]. This heterocycle (Fig. 2) bears some similarity to the ligand structure of Avid AL gel (Fig. 2), which has been used for the purification of IgG [31]. Again, there seems to be no correlation between the presence of this reactive group and a higher capacity for IgG (see Fig. 1).

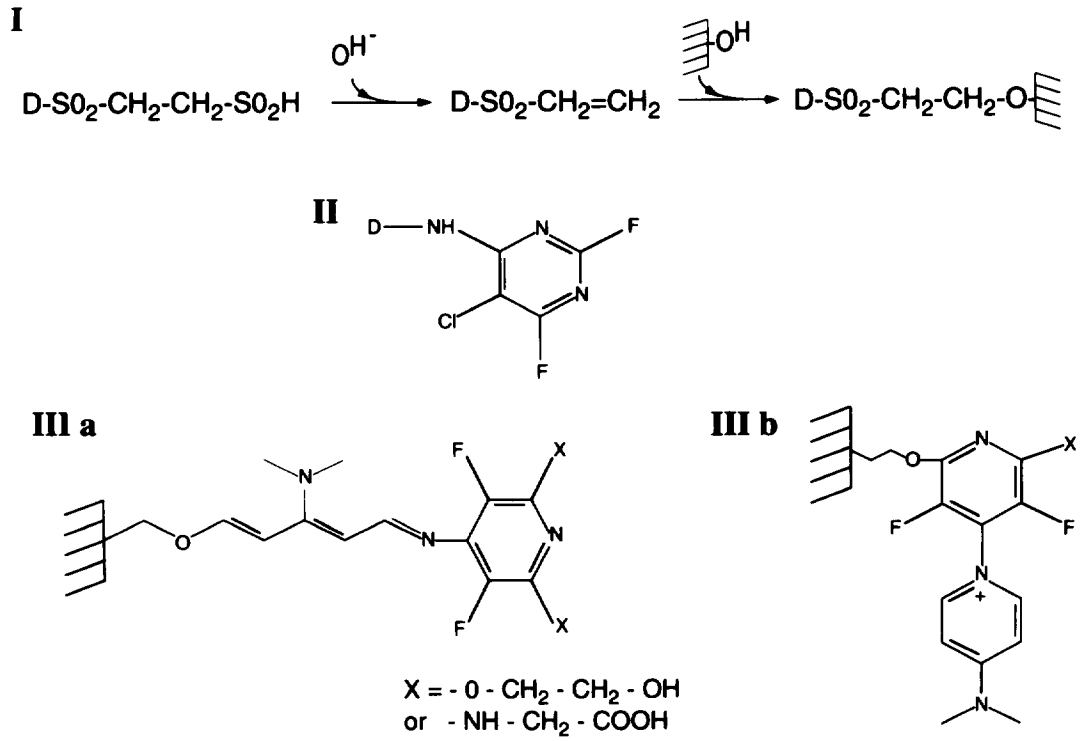

Fig. 2. Panel I, reactive group of Remazol dyes. Sulfate esters of hydroxyethyl sulfonyl dyes are converted to the reactive vinyl sulfonic derivative by mildly alkaline condition [29]. Panel II: Reactive group of Drimarene dyes [29]. Panel III: Structures of the Avid AL ligand [31].

Hence, the chromophoric group probably plays a prominent role in the affinity of the dyes for IgG.

3.4. Selectivities demonstrated by some immobilized dyes

This work was undertaken with the hope of detecting immobilized dyes with a higher affinity for IgG2 than for IgG1. Such a dye would allow us to load the column with total IgG and to displace the more abundant IgG1, with IgG2 present at a lower concentration from the dye column. An immobilized dye with such a property could be used in a column of a lower volume compared to that necessary when packed with a dye with a higher affinity for IgG1 than for IgG2. Such a favourable condition has been encountered previously for the purification of a quantitatively minor erythrocytic enzyme that could displace hemoglobin present in the starting material at a 5600-fold higher concentration from a dye column [32]. The present study did not detect a dye with a higher affinity for IgG2 than for IgG1.

In fact, most of the dyes showed no interesting selectivity towards IgG subclasses. ELISA for IgG2 and total IgG in the first fractions collected during the IgG breakthrough did not show an interesting deviation from the proportion present in the incoming solution. Data for one such dye (A3) are shown in Fig. 3.

From the 69 dyes tested, six immobilized dyes (R1, R6, R17, Y8, R5 and Y5) were found to display a significantly lower affinity for IgG2 than for IgG1, as reflected by a high IgG2–total IgG ratio in the first fractions of the IgG breakthrough (examples are shown on Fig. 3). In addition of their interesting selectivity for IgG, R6 and Y8 also have a significant capacity for IgG (Figs. 3 and 1).

Fig. 3 also demonstrates that a higher degree of

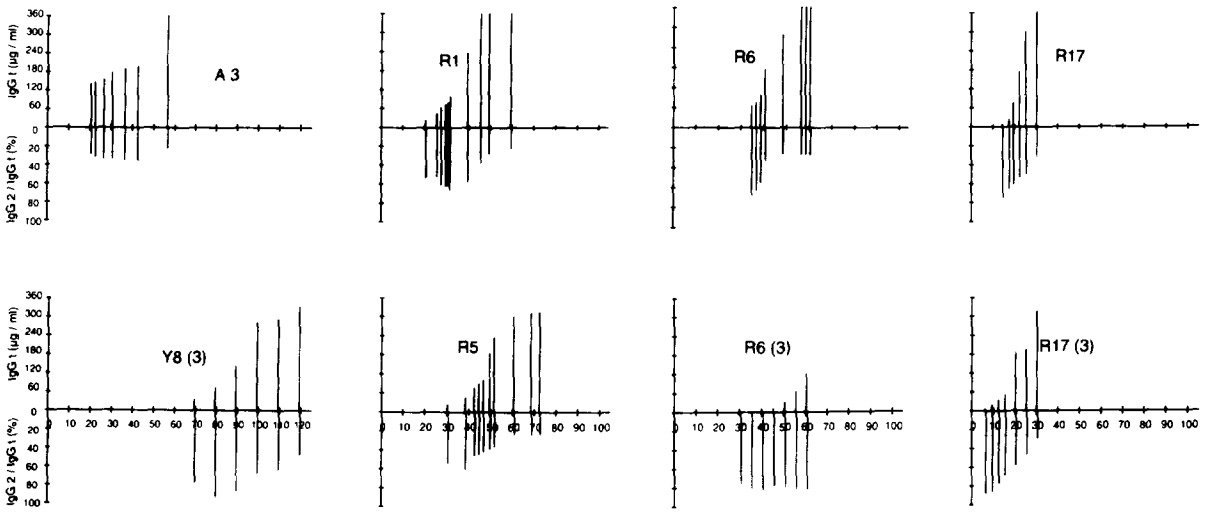

Fig. 3. Studies on the selectivities of various dyes by frontal chromatography (only examples are shown but every dye of Fig. 1 has been studied in a similar manner). The abscissa shows the fraction numbers (0.5 ml/fraction). The upper ordinate gives the amount of total IgG (IgGT) in the assayed fractions. The concentration of IgGT in the starting material was 0.63 mg/ml. The lower ordinate gives the ratio of IgG2–total IgG in the fractions collected. The value of the IgG2–IgGT ratio in the starting material was 28%. Dye A3 does not show any interesting selectivity for the separation of IgG subclasses, as the percentage of IgG2–IgGT in the first fractions after protein breakthrough are too close to the value found in starting material. In contrast, the proportion of IgG2 is significantly increased in the first protein-containing fractions eluted from the other dyes, hence, they may be considered as efficient dyes for the preparation of IgG2-enriched IgG solutions (see text).

dye substitution did not alter dye selectivity (compare data for R6 and R6(3) and for R17 and R17(3)).

3.5. Evaluation of the immobilized Procion Yellow HE-4R used to prepare IgG2-enriched IgG solutions

Results of frontal screening led us to investigate the use of one of the better performing dyes, Y8 (Procion Yellow HE-4R) for the preparation of IgG2-enriched IgG solutions.

Frontal chromatography was clearly ineffective for this purpose. Fractions with a high level of purity could be pooled (i.e. the first fractions collected at the breakthrough of IgG), in order to prepare an IgG2-enriched IgG preparation, however, the yield would be extremely low and a better yield might be obtained only at the expense of purity. Hence, experiments have been devised (see Section 2) in order to find optimal mobile phases for the use of Y8 in zonal mode: The aim was to find eluents that were capable of selectively eluting IgG2 retained on the Y8 immobilized dye.

3.6. Screening of satisfactory eluents for zonal chromatography of IgG on Procion Yellow HE-4R

In order to evaluate the merits of zonal chromatography for the purification of IgG2, a Y8 column was loaded with total IgG without exceeding its capacity (4 mg of IgG per ml of gel were used). The results obtained, in terms of yield and purity of IgG2 with various eluents, are diagramatically shown in Fig. 4. Aimed targets for yield and purity were realistically set at 40 and 60%, respectively.

A moderate ionic strength increase, obtained by the addition of NaCl to the mobile phase (up to 0.2 *M*, Fig. 4), eluted some IgG2 with low yield and only marginal enrichment.

Modification of the pH of the mobile phase was checked as a means of preparing IgG2-enriched fractions. Fig. 4 shows that when the pH of the mobile phase is increased, some IgG2 is eluted from

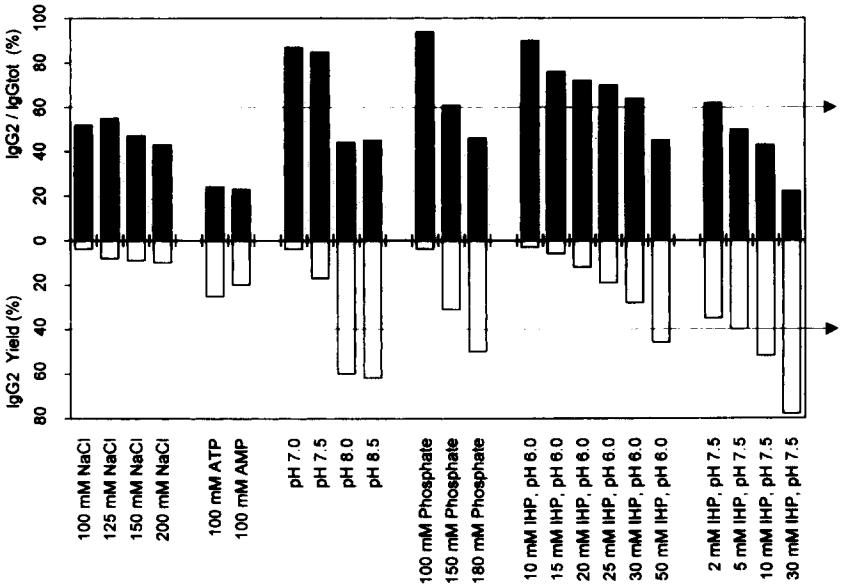

Fig. 4. Screening of mobile phase conditions to find satisfactory selective desorption procedures for IgG2. The composition of each of the mixtures studied is written in abbreviated form at the bottom (if not otherwise indicated, tested eluent mixtures were adjusted to pH 6.0, see Section 2 for full details). The performance of each of the mixtures studied is represented by paired bars on the histogram: The lower bar gives the yield of IgG2 present in the eluted pool and the upper bar shows the IgG2–total IgG ratio, expressed as a percentage. The lower arrow indicates the target value for yield while the upper arrow indicates the target value for IgG2 enrichment.

the column. Increasing the pH has been recognized since the early days of immobilized dye chromatography as an efficient means of eluting retained proteins [22,33]. Some selectivity was observed since, at pH 7.5, the IgG2–total IgG ratio was increased to 85%, however, the yield was rather low (17%).

Phosphate was shown to elute IgG2 from the immobilized Procion Yellow HE-4R column: 180 m*M* phosphate eluted about 50% of the retained IgG2. Lower concentrations did elute less IgG and satisfactory selectivity could be obtained. The demonstrated selectivity of phosphate contrasts with the results observed with sodium chloride and suggests that the eluting strength of phosphate is not due merely to the increase in ionic strength. It has been shown that inclusion of phosphate in the mobile phase dramatically affected the capacity of immobilized dye columns for enzymes with phosphorylated substrates or cofactors [34]. A specific eluting effect of phosphate ions has also been demonstrated with enzymes belonging to other classes (e.g., Ref. [35]).

It has been amply demonstrated that screening several substances (such as phosphorylated sugars, nucleotides, amino acids) could lead to the finding of eluents with a satisfactory selectivity for proteins retained on a dye column [32,36–38]. It is assumed that these substances compete with dye–protein interaction since, as dyes, they possess charged substituents with a defined space distribution, and eventually an aromatic backbone. Hence, some of these potential eluents were evaluated as a means of eluting IgG2 with both a satisfactory yield and adequate purity:

ATP and AMP were useless, as concentrations as high as 100 m*M* eluted only 25 and 20% of total IgG, respectively, and no IgG2 enrichment was obtained. Results obtained with IHP were distinctly better in terms of yield and purity but, nevertheless, fell short of pre-established limit values (Fig. 4).

The better results obtained in terms of IgG2 enrichment, both with IHP and mobile phase pH variations, led us to check if combining the addition of IHP to the mobile phase and the use of a higher pH value would be valuable. Results obtained by dissolving IHP at various concentrations in mobile

phase (pH 7.5) are shown in Fig. 4. Again, it was not possible to obtain IgG2 with a purity greater than 60% at a yield that was not less than 40%.

3.7. IgG2 enrichment of igG solutions on immobilized Procion Yellow HE-4R

Based on the results of screening experiments, we devised a procedure that produced IgG solutions that were adequately enriched with IgG2 and had a satisfactory yield. This was achieved by performing two passes on the same Y8 column used for IgG2 elution, increasing the pH of the mobile phase for the first pass, and using an IHP concentration gradient for the second pass, as described in Section 2. The ELISA performed on fractions collected during the second pass (Fig. 5) allowed us to pool fractions so as to obtain an IgG solution complying with preset specifications. The results obtained were 43 and 67% for cumulative yield and IgG2/total IgG, respectively (Fig. 5).

4. Conclusion

The results obtained with Procion Yellow HE-4R may lay the basis for a preparative procedure for IgG2-enriched IgG solutions, which are needed for therapeutic applications. With this in mind, it must be recalled that immobilized dyes are already used frequently for the preparation of proteins for therapeutic use. However, attention has to be paid to problems such as leachable levels in column effluents and possible leachable toxicity (which has, in fact, proven to be low for the dyes studied to date [39,40]).

This study exemplified the power of a frontal screening procedure for discovering useful dyes for

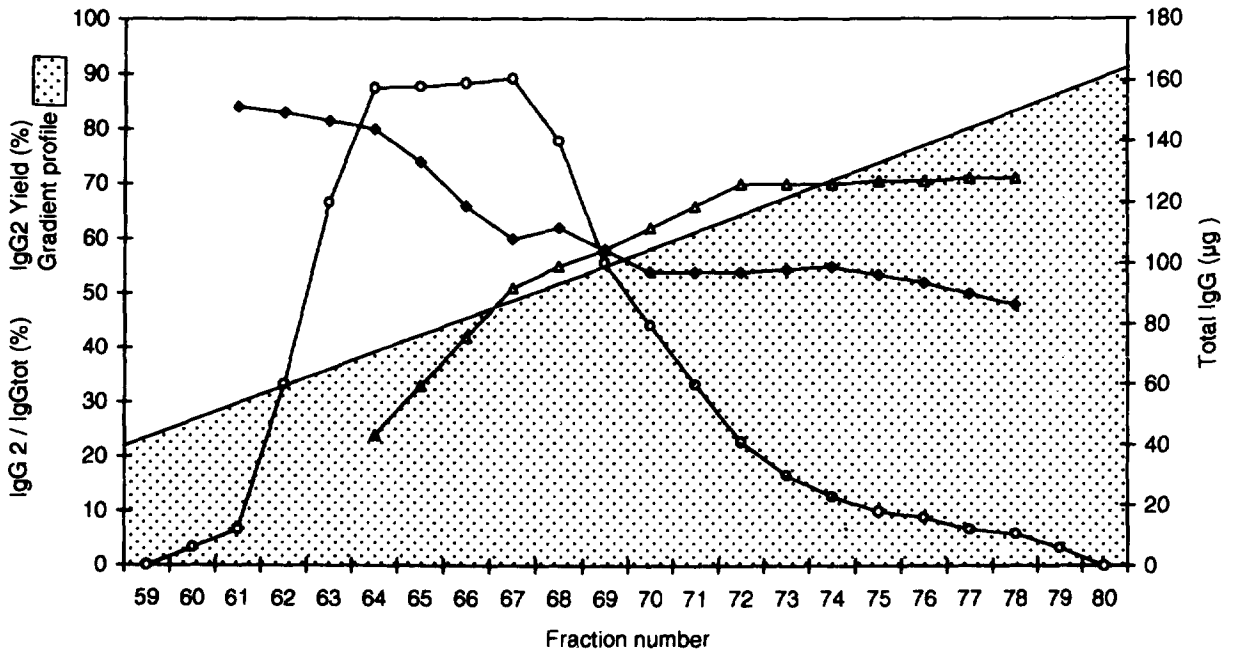

Fig. 5. Preparation of an IgG2-enriched IgG solution by chromatography on immobilized Procion Yellow HE-4R. IgG-containing fractions eluted by buffer at pH 8 in the first chromatographic run on immobilized dye were pooled. The pH value was adjusted to 6.0 and the pool was loaded again on the same dye column. The column was then developed with an IHP concentration gradient from 0 to 100% limit buffer (50 m*M* imidazole in buffer A). The profile of the gradient that was pumped to the column during collection of fractions 58 to 80 is shown by the dotted area. IgG2 (empty circles) and total IgG were assayed in all fractions. Values for IgG enrichment (ratios of IgG2–total IgG are expressed as percentages) and cumulative yields are shown by empty triangles and filled lozenges, respectively. Fractions 60 to 68 were pooled in order to comply with preset specifications, i.e., a yield of not lower than 40% and an IgG2–total IgG ratio of not lower than 60%. The actual values for yield and enrichment in the pooled fractions (60 to 68) were 43 and 67%, respectively.

IgG2 purification. Again, systematic screening of some eluting conditions was fruitful and allowed the identification of an efficient and low cost eluent.

Acknowledgments

We thank the dye manufacturers for the samples of dyes used in this study.

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