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Adsorption of human serum proteins onto TREN-agarose: Purification of human IgG by negative chromatography

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ABSTRACT

Tris(2-aminoethyl)amine (TREN) – a chelating agent used in IMAC – immobilized onto agarose gel was evaluated for the purification of IgG from human serum by negative chromatography. A one-step purification process allowed the recovery of 73.3% of the loaded IgG in the nonretained fractions with purity of 90–95% (based on total protein concentration and nephelometric analysis of albumin, transferrin, and immunoglobulins A, G, and M). The binding capacity was relatively high (66.63 mg of human serum protein/mL). These results suggest that this negative chromatography is a potential technique for purification of IgG from human serum.

1. Introduction

Human immunoglobulin G (IgG), the major class of serum glycoproteins, constitutes an important therapeutic protein for a number of malignancies [1] and is also required for immunodiagnostic and immunochromatographic (downstream-processing) purposes. Therefore, there is a high demand for high purity IgG, free from other serum proteins. Processes for the purification of human IgG typically involve precipitation with ethanol followed by chromatographic techniques (such as ion exchange chromatography, gel permeation chromatography, or affinity chromatography) [2,3].

The proteins A, G, and L are the most frequently biological specific ligands used in affinity adsorption for IgG. Due to their high affinity for the Fc antibody domain (proteins A and G) and the variable domain of the human kappa chain (protein L), these ligands can be employed in a one-step adsorption process for IgG purification or extracorporeal removal of autoimmune IgG from biological fluids [4–9]. However, protein A, G, and L adsorbents are expensive and the desorption of IgG involves drastic and denaturing elution conditions and cannot withstand the harsh conditions of cleaning procedures. In addition, these proteins are susceptible to degradation and leakage after some purification cycles [1,10]. These drawbacks have stimulated the development of more stable and less expensive adsorbents in recent years.

Pseudospecific affinity ligands such as nonbiological molecules (metal chelates, thiophilic, and dye ligands) and biological molecules (like the amino acid histidine) have been studied for human IgG purification by many research groups [11–16]. Generally, the pseudobiospecific ligands are low cost and have high stability, capacity, simplicity, and selectivity [17]. Among these ligands, the immobilized amino acid histidine is an interesting alternative for human IgG purification [18,19]. As an example, histidine grafted aminohexyl-Sepharose 4B was used as a negative affinity adsorbent for purification of IgG from human plasma diluted 20 times in Mops buffer at pH 7.2 [20]. The negative chromatographic mode (as cited by many authors [21–25]) aims at allowing the product to pass through the column, retaining only the contaminants or impurities [26]. The adsorption of serum proteins in histidine grafted aminohexyl-Sepharose seems to be due to the electrostatic interaction of albumin and other proteins of human serum by the free remaining cationic α-NH_3^+ of histidine. The unprotonated imidazole group of histidine is also involved in the retention of serum protein around neutral pH [20].

The nonbiological ligand TREN (Tris(2-aminoethyl)amine) is a quadridentate chelating ligand used in IMAC with four nitrogen atoms, three of which are primary in nature and the fourth one is tertiary. TREN chelated with copper and nickel ions has been employed in protein purification [16,27–29]. Due to its high amine residue content, TREN (without chelated metal ion) can serve as

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an anion exchanger. At a pH lower than 10.0, TREN is positively charged (according to the manufacturer, Sigma–Aldrich) and can adsorb negatively charged molecules, so, this ligand could be an excellent candidate for the purification of IgG from serum proteins. However, in the literature there is no study describing TREN as a ligand for IgG purification.

Therefore, the purpose of this study is to evaluate the feasibility of using the ligand TREN immobilized onto agarose through an ether linkage (epichlorohydrin-activated gel) for the purification of IgG from human serum by negative chromatography. The main advantages of this unconventional proposed process is the use of nonbiological ligand that costs less and is more stable than ligands traditionally used in affinity chromatographies. Experimental studies were conducted aiming to find the least favorable conditions – pH and buffer system (phosphate, Tris–HCl, Bis–Tris, Mes, and Mops) – for human IgG adsorption. The breakthrough curve and dynamic capacity of serum proteins were determined since they are the basis for process design, scale-up, and optimization of large-scale negative chromatographic mode separation processes.

2. Experimental

2.1. Materials

The TREN-agarose gel (cross-linked 4% beaded agarose, activated with epichlorohydrin), 3-(N-morpholino)propanesulfonic acid (Mops), bis(2-hydroxyethyl)amino–tris(hydroxymethyl) methane (Bis–Tris), crystalline bovine serum albumin (BSA), and human serum albumin (HSA, 98–99% purity) were obtained from Sigma (USA). The electrophoresis calibration kit for molecular mass determination (myosine, 210 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 110 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa) was provided by GE Healthcare (USA). Tris(hydroxymethyl amino methane) and 2-(N-morpholino)ethanesulfonic acid (Mes) were purchased from Merck (Germany). Prepurified human immunoglobulin G (containing 98.3% IgG according to the nephelometric analysis of IgG, IgM, IgA, albumin (HSA), and transferrin done in our laboratory) was provided by Aventis Behring (Germany). The nephelometric reagents were purchased from Beckman Coulter (USA). The water used in all experiments was ultrapure water obtained using a Milli-Q System (Millipore, USA). All other chemicals were of analytical reagent grade.

2.2. Preparation of human serum

Blood samples from a healthy donor were collected without anticoagulant. These samples were centrifuged at 4 °C for 5 min at 1000 × *g* and the supernatant was used without further treatment. To ensure that proper ionic strength and pH were maintained for optimal binding, it was necessary to dilute serum samples with the proper adsorption buffer.

2.3. Analysis of serum proteins

Total protein concentration was determined by the Bradford method [30] using BSA as reference protein. The concentrations of IgG, IgA, IgM, HSA, and transferrin in the fractions collected in the chromatographic experiments were determined nephelometrically using an Array Protein System (Beckman Coulter, USA), in accordance with the method provided by the manufacturer.

2.4. Sodium dodecyl sulfate polyacrylamide gel elecrophoresis (SDS-PAGE) and isoelectrofocusing (IEF)

The chromatographic fractions were analyzed by SDS-PAGE (7.5% polyacrylamide gels) under nonreducing conditions using a Mini-Protean III System (Bio-Rad, USA) in accordance with Laemmli [31] and stained with silver nitrate in accordance with Morrissey [32]. The PhastSystem (Pharmacia, Sweden) and pH 3–9 gradient gels (GE Healthcare, USA) were used for IEF and stained with silver nitrate in accordance with the method provided by the manufacturer.

2.5. Chromatographic experiments

All chromatographic procedures were carried out with an automated chromatography system (Econo Liquid Chromatography System, Bio-Rad, USA) at 25 °C at a flow rate of 0.5 mL min^{-1}. For studies concerning the influence of the buffer on human serum protein adsorption, the following loading buffers at 25 mM covering a pH range from 5.5 to 9.0 within their respective buffering ranges were used: Mops, Mes, Tris–HCl, Bis–Tris–HCl, and sodium phosphate.

The TREN-agarose gel was suspended in the loading buffer described above, degassed, and packed into columns (10.0 cm × 1.0 cm I.D., GE Healthcare, USA) to give bed volumes of approximately 1.0 or 3.0 mL.

Human serum (0.1 or 0.3 mL) and its solutions diluted 5, 10, or 20 times with an appropriate loading buffer were injected into the column, which had been previously equilibrated with loading buffer. For the experiments with prepurified human IgG, protein samples containing 2.0 mg of IgG diluted in 2.0 mL of equilibration buffer was loaded into the column (bed volume of 3.0 mL). For both experiments, after protein injection, the column was washed with loading buffer until the absorbance values at 280 nm of eluate were close to 0. Elution was performed with the loading buffer containing 0.4 M NaCl.

During the wash steps and elution, absorbance at 280 nm was monitored and fractions of 1.0 and 2.0 mL, respectively, were collected. The Bradford method [30], nephelometric analysis, SDS-PAGE, and IEF were used to analyze the proteins in nonretained and retained fractions. After each experiment, the column was washed with 50 mM NaOH, followed by water, and finally by the loading buffer to restore it to its initial conditions (regeneration) for carrying out the next experiment.

2.6. Adsorption breakthrough curves

These experiments were carried out at 25 °C with an automated chromatography system (Econo Liquid Chromatography System, Bio-Rad, USA). After equilibration of TREN-agarose with the loading buffer (25 mM Mes buffer, pH 6.5), human serum diluted 20 times with the loading buffer was pumped through the column at a flow rate of 0.5 mL min^{-1} (residence time, t_R of 36 s, calculated by dividing the bed interstitial volume by the flow rate). The column outlet absorbance at 280 nm was continuously monitored. The loading of the protein solution was stopped when absorbance at 280 nm at the column outlet became constant after an initial increase. The unabsorbed protein was washed out of the column with loading buffer. The adsorbed proteins were eluted with a 25 mM Mes buffer, pH 6.5, containing 0.4 M NaCl. The effluents were monitored as described previously (measurement of absorbance at 280 nm). After elution had been completed, the column was regenerated by sequentially washing with 50 mM NaOH, followed by water and the loading buffer.

Protein concentrations in the retained and nonretained fractions were determined by the Bradford method [30] and nephelometric analysis and analyzed by SDS-PAGE under nonreducing conditions. Breakthrough curves were plotted as the ratio of the total protein concentration at the outlet (C) to that in the feed stream (C_0) as a function of the volume of protein solution throughput.

2.7. Batch adsorption of human serum proteins, HSA, and human IgG on TREN-agarose gel

Experiments for the determination of human serum proteins, HSA, and human IgG adsorption isotherms on TREN-agarose gel at 25 °C were carried out (in duplicates) in Eppendorf tubes (as stirred tanks) with 50 μL of gel. The gel was previously equilibrated with degassed 25 mM Mes buffer at pH 6.5 and the aliquots of 1.0 mL of human serum, HSA, or IgG solutions were added to the tubes. The initial protein concentrations of HSA, IgG, and human serum were in the range of 0.5–50.0 mg/mL, 0.5–20.0 mg/mL, and 0.5–12.2 mg/mL, respectively. The tubes were agitated for 3 h to allow equilibrium to be established. After this, protein equilibrium concentration in the liquid phase (C^*) was quantified. For single systems (HSA and IgG), the concentration of protein was measured based on absorbance at 280 nm (UV–vis spectrophotometer, Beckman DU 650, USA). For human serum proteins, the total protein concentration was determined with the Bradford method [30]. The difference in the unbound protein concentrations between the experiments did not exceed 2.0%, 0.5%, and 1.5% for IgG, HSA, and human serum, respectively. The mass of protein adsorbed per volume of gel (mg/mL), Q, was calculated as the difference between the amount of protein added and the one remaining in the liquid phase after equilibrium divided by the volume of the adsorbent. Plotting Q as a function C^* yielded the equilibrium isotherm. Langmuir isotherm model [33] (Eq. (1)) was used to fit the data:

$$Q = \frac{Q_m C*}{K_d + C*} \quad (1)$$

in which Q_m is the maximum protein binding capacity and K_d is the dissociation constant, which represents the affinity between protein and adsorbent. Values of K_d and Q_m were estimated from a nonlinear least-squares (Levenberg–Marquardt) fit of Langmuir model to the data.

3. Results and discussion

3.1. Effect of buffer system on serum protein adsorption

In order to select the best loading buffer for IgG purification by negative chromatography, chromatographic experiments were carried out by loading 0.1 mL of human serum diluted 20 times with four loading buffers at different pHs (Mops, Mes, Tris–HCl, and Bis–Tris) onto 1.0 mL of TREN-agarose column. After washing the nonretained proteins, the buffer was changed to loading buffer containing 0.4 M NaCl. Retained and nonretained fractions were analyzed by the Bradford method [30], nephelometry, and SDS-PAGE. It was expected that the impurities would adsorb onto the matrix, while IgG would be collected in the nonretained (flow-through and washing) fractions. The results were evaluated in terms of IgG recovery in the flow-through and washing streams and of selectivity for IgG in order to determine the one that is least favorable for IgG adsorption.

3.1.1. IgG recovery in nonretained fractions

IgG recovery in the flow-through and washing streams was found to depend on pH and the nature of the buffer ions (Fig. 1).

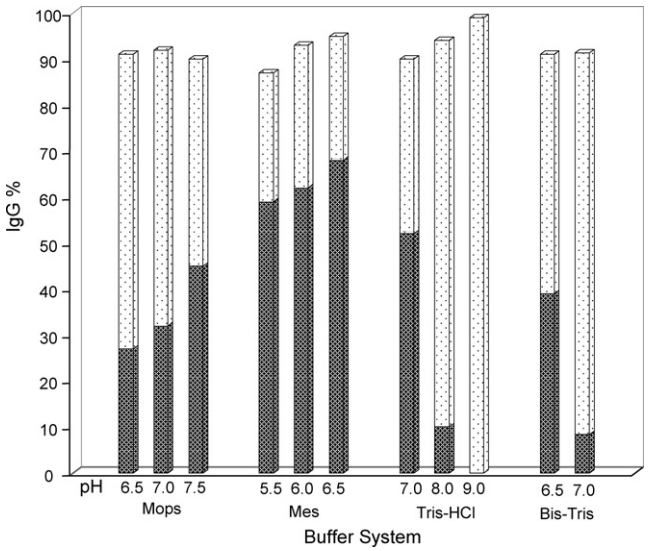

Fig. 1. Effect of buffer system and pH on retention and nonretention of human IgG by TREN-agarose adsorbent. The retained and nonretained fractions were analyzed for IgG by nephelometry. (▩) % of nonretained IgG; (⬚) % of retained IgG.

Nonretention of IgG was observed between pH 5.5 and 8.0 irrespective of the buffer used. For the case of Tris buffer at pH 9.0, around 99% of the loaded IgG was retained under this condition. The most favorable pH value range for the separation of IgG with negative chromatography was from 5.5 to 7.5, being the specific pH value depended on the type of buffer employed. For example, approximately the same amount of IgG was nonretained in Mops buffer at pH 7.5 (0.44 mg) as in Bis–Tris at pH 6.5 (0.39 mg).

The interactions between the adsorbent and human IgG seemed to be mainly electrostatic interactions between IgG and amino charged groups of immobilized TREN. According to the manufacturer, the charge on the TREN-agarose is zero at pH values around 10.5 and fully charged at pH values around 4.5. The gel is probably positively charged at around pH 9.0. Then, in the pH range of 5.5 to 7.5, the TREN-agarose had a strong positive charge which created a repulsion for the IgG molecules (pI in the range of 5.0–9.0). As the pH was increased from 7.5 to 9.0, the IgG molecules gradually lost their positive charge, resulting in a higher IgG adsorption.

Thus, the conditions least favorable for IgG adsorption of each buffer studied (Mes pH 6.5, Mops pH 7.5, Tris–HCl pH 7.0, and Bis–Tris pH 6.5) were chosen to evaluate the selectivity of the adsorbent.

3.1.2. Selectivity of the adsorbent

The effect of the nature of the buffer ions on IgG purification by negative chromatography was examined (Fig. 2 and Table 1). For all buffers studied, the serum proteins were efficiently captured with the TREN-agarose adsorbent. The SDS-PAGE showed that the purified IgG from human serum obtained in unbound fractions had a purity similar to that of the commercial IgG product (Aventis Behring). Under all experimental conditions tested, the amount of nonretained HSA, IgM, and IgA was lower than the Array Protein System detection range. The purity of IgG in the nonretained fractions was in the range of 90–95%, based on IgG and total protein concentrations determined with nephelometric and Bradford [30] methods, respectively. However, the highest IgG recovery in nonretained fractions was obtained with Mes buffer, pH 6.5 (68%).

The optimum combination of yield and purity of the IgG recovered from human serum with TREN-agarose adsorbent was similar to the results for a biomimetic ligand immobilized in agarose reported by Teng et al. [34]. They used this synthetic bifunctional

Table 1
IgA, IgG, IgM and HSA recovery after chromatographic purification of human serum on TREN-agarose gel (human serum diluted 20 times).

Buffer	pH	Injection (mg)				Unbound fractions (mg)				Bound fractions (mg)				Unbound (%)	Bound (%)
		IgG	IgA	IgM	HSA	IgG	IgA	IgM	HSA	IgG	IgA	IgM	HSA	IgG	IgG
Mes	6.5	1.23	0.21	0.15	3.54	0.84	0.00	0.00	0.00	0.33	0.20	0.13	3.38	68	27
Mops	7.5	0.98	0.06	0.08	3.36	0.44	0.00	0.00	0.00	0.33	0.00	0.06	3.27	45	34
Tris–HCl	7.0	1.12	0.07	0.11	3.42	0.59	0.00	0.00	0.00	0.39	0.06	0.07	3.80	52	35
Bis–Tris	6.5	1.50	0.15	0.18	4.48	0.59	0.00	0.00	0.00	0.32	0.15	0.08	3.01	39	21

ligand to purify IgG from human plasma. The retained human IgG was eluted with glycine–HCl buffer pH 2.7–2.9 with recovery of 60–69% and purity of 92.5–99.2% [34].

The adsorption behavior of serum proteins varied in TREN-agarose, depending on the buffer system. When phosphate buffer was tested at pH 6.0 and 8.0 for IgG purification by negative chromatography, the serum proteins were partially adsorbed and IgG was obtained in nonretained fractions contaminated with several serum proteins (data not shown). This may be the result of the electrostatic interaction of phosphate for the immobilized TREN (phosphate buffer has a negative charge and TREN has a positive charge), masking the binding site of the ligand molecule.

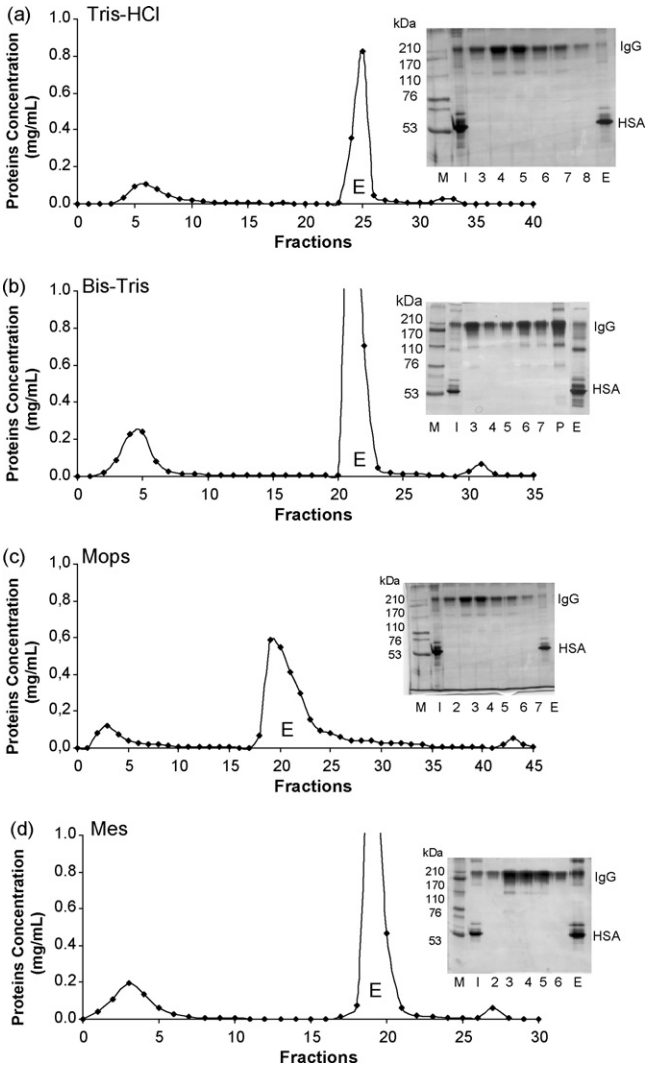

Fig. 2. Effect of the nature of the buffer ions on purification of IgG from human serum diluted 20 times by negative chromatography. (a) Tris–HCl buffer, pH 7.0; (b) Bis–Tris buffer, pH 6.5; (c) Mops buffer, pH 7.5; (d) Mes buffer, pH 6.5. Nonreducing SDS-PAGE analysis of fractions from chromatography on TREN-agarose gel: M, molecular mass protein marker; I, injected human serum solution; Numbers in SDS-PAGE correspond to the nonretained fractions indicated in chromatograms; E, pool of retained fractions (eluted with the adsorption buffer containing 0.4 M NaCl); P, human IgG standard (Aventis Behring).

The nature of the interaction between TREN-agarose and human IgG has yet to be established. IgG adsorption seemed to be governed by electrostatic forces since elution was possible with salt (NaCl). In order to verify this hypothesis the isoelectric points (p*I*) of the retained and nonretained IgG fractions were determined by isoelectrofocussing (electrophoresis not shown). The results showed p*I* from 5.8 to 9.0 for the prepurified IgG used for the experiments, a presence of molecules of p*I* in the range of 5.8–7.3 in the retained fraction and proteins with p*I* from 6.3 to 9.0 in the nonretained pool. These results are in accordance with the electrostatic mode of interaction proposed above. However, the present data alone does not allow a full understanding of the interactions between the TREN-agarose gel and the other serum proteins. A precise understanding is made difficult by the heterogeneous nature of the system.

Protein adsorption depends very much on nature of the adsorbent and the protein containing in sample; the target protein can be partially or completely nonretained in charged adsorbent. Qi et al. [35] studied a chromatographic method tandem system (coupled DEAE anion-exchange and Protein G affinity columns) for simultaneous separation and purification of IgG and albumin from mouse serum. Under neutral conditions, DEAE column adsorption most of the acidic proteins; mouse IgG was completely unretained on the column (with few contaminants), and subsequently captured at the Protein G column, and eluted with high purity [35].

Although human serum proteins in Mes buffer at pH 6.5 showed effective binding to TREN-agarose, an optimization of the feedstream dilution is required in order to establish a simple one-step procedure for the purification of IgG from human serum by negative chromatography.

3.2. Effect of feedstream dilution

In order to define feedstream dilution, chromatographic experiments were performed on TREN-agarose under the same conditions used for human serum "in natura" (conductivity of 9.21 mS/cm, pH 7.4) and human serum diluted 5 (conductivity of 3.02 mS/cm, pH 6.9) and 10 times (conductivity of 2.10 mS/cm, pH 6.7) with 25 mM Mes buffer pH 6.5.

Table 2
Total protein recovery after chromatographic purification of human serum on TREN-agarose gel.

Pool fractions	Dilution factor							
	0 time		5 times		10 times		20 times	
	mg	%	mg	%	mg	%	mg	%
Injection	15.93	100.0	11.25	100.0	16.50	100.0	15.14	100.0
Unbound	7.82	49.1	2.09	18.6	1.84	11.1	2.79	18.4
Bound	9.78	61.4	7.66	68.2	14.37	87.0	14.92	98.6
Regeneration	0.00	0.0	0.04	0.4	0.22	1.3	0.06	0.4
Recovery	17.61	110.6	9.79	87.1	16.44	99.6	19.04	117.0

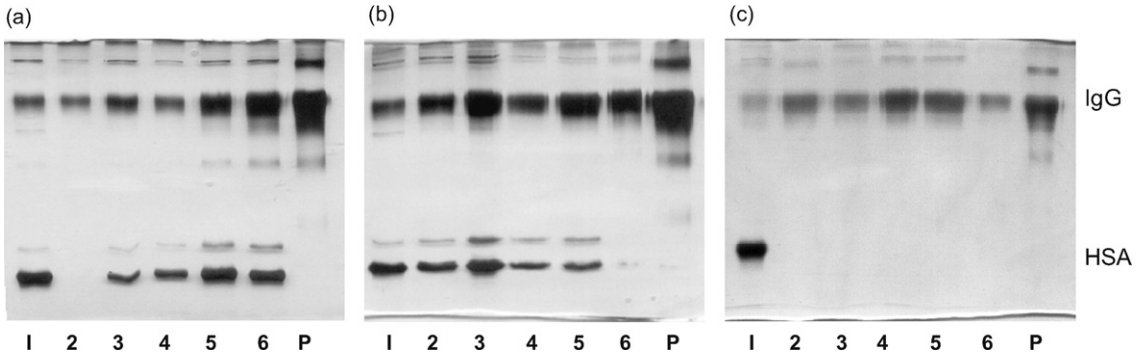

Fig. 3. Effect of dilution factor of human serum in IgG purification by negative chromatography in TREN-agarose. Nonreducing SDS-PAGE of (a) human serum "in natura"; (b) human serum diluted five times; (c) human serum diluted ten times. I, injected human serum solution; numbers in SDS-PAGE correspond to the nonretained fractions; P, human IgG standard (Aventis Behring).

Table 3
Nephelometric analysis of IgA, IgG, IgM, HSA, and transferrin after chromatographic purification of human serum on TREN-agarose (human serum diluted ten times).

Fractions	IgA		IgG		IgM		HSA		Transferrin	
	mg	%	mg	%	mg	%	mg	%	mg	%
Injection	1.12	100.0	4.32	100.0	0.44	100.0	12.90	100.0	0.77	100.0
Unbound	0.00	0.0	3.17	73.3	0.00	0.00	0.00	0.00	0.00	0.00
Bound	1.05	94.3	1.09	25.2	0.44	100.6	13.66	105.9	0.74	96.3
Recovery	1.05	94.3	4.25	98.4	0.44	100.6	13.66	105.9	0.74	96.3

Retained and nonretained fractions were analyzed with the Bradford method for protein concentration and with SDS-PAGE for purity (Table 2, Fig. 3). The results were compared with experiments carried out with human serum diluted 20 times (conductivity of 1595 μS/cm, pH 6.5), shown in Fig. 2d. Serum proteins retention on TREN-agarose was affected by conductivity and pH of the medium. TREN-agarose gel adsorbed considerable amounts of HSA and other serum proteins (Fig. 3), but high purity IgG was obtained only when the serum was diluted at least 10 times. The binding capacity of the gel for serum proteins increased with feedstream dilution: the protein capture was more efficient at lower conductivity and pH values closer to 6.5. These results are in accordance with the electrostatic mode of interaction of classical ion-exchangers in which dilution of serum or plasma is necessary for high adsorption due to high conductivity of these fluids.

73.3% of the loaded IgG was obtained in nonretained fractions when a 3.0 mL bed volume column of TREN-agarose was loaded with 0.3 mL of human serum diluted ten times (Table 3). It was shown that nonretained fractions have high purity (based on SDS-PAGE analysis and IgG, IgM, IgA, HSA, and transferrin nephelometric analysis), since a purity similar to that of the commercial IgG product was achieved (Aventis Behring).

3.3. Adsorption breakthrough curve

The dynamic capacity of TREN-agarose was estimated by overloading a 1.0 mL bed volume column with human serum diluted

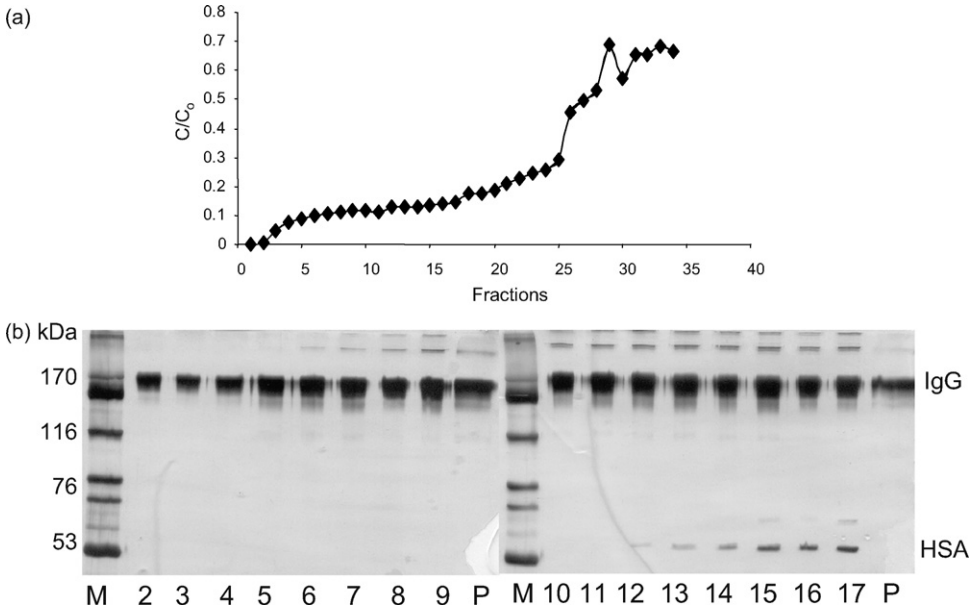

Fig. 4. (a) Breakthrough curve of human serum solution (diluted 20 times) for TREN-agarose gel. (b) SDS-PAGE analysis under nonreducing conditions of fractions of breakthrough curve: M, molecular mass protein marker; 2–17, nonretained fractions; P, human IgG standard (Aventis Behring).

20 times in Mes buffer, pH 6.5 (3.50 mg mL^{-1} (119.00 mg) of total protein and 0.58 mg mL^{-1} (19.72 mg) of IgG) at a flow rate of 0.5 mL min^{-1}. Fig. 4a displays the breakthrough curves quantified by the Bradford method (the unbound fractions were analyzed with SDS-PAGE (Fig. 4b)). Initially, the IgG band (molecular mass of approximately 150 kDa) was detected by SDS-PAGE, leading to the conclusion that the other concentration of proteins at the outlet was zero, reflecting complete adsorption of the serum molecules onto the TREN-agarose. As the loading step proceeded and binding sites became occupied, HSA was detected at the outlet (after fraction #12). Defining the breakthrough point as the fraction in which HSA is detected by SDS-PAGE, a volume of 12.0 mL of human serum solution was loaded prior to breakthrough (approximately 7 mg of IgG unbound mL^{-1} of matrix). Similar results were obtained by Pitiot et al. [20] for purification of IgG from human plasma on histidine grafted aminohexyl-Sepharose 4B used as a negative affinity adsorbent. The authors obtained 6.46 mg IgG (corresponding to 72.6% of the injected IgG) in nonretained fractions when 1.0 mL of human plasma diluted 20 times in Mops buffer at pH 7.2 was fed into the column.

In a large-scale purification process, it is desirable to minimize product loss during the loading step. Consequently, the loading of feed is allowed to proceed until breakthrough occurs, at which point the loading step is terminated [36] (in this case, loading 12.0 mL of human serum solution).

The contaminant proteins that were observed in the pass-through fractions after 12.0 mL could be removed by reprocessing of the nonretained fractions. SDS-PAGE analysis showed that pure IgG fractions could be obtained after the second run (data not shown).

After washing, adsorbed proteins were eluted with Mes buffer, pH 6.5 containing 0.4 M NaCl and protein concentration in the eluted fractions was determined by the Bradford method and analyzed by SDS-PAGE. The dynamic capacity of TREN-agarose under this condition was 31.65 mg of serum proteins/mL of gel. The protein profile given by SDS-PAGE analysis of eluted fractions from the breakthrough experiment was similar to the one verified for eluted samples from negative chromatography experiments carried out by loading 0.1 mL of human serum diluted 20 times (data not shown).

3.4. HSA, human IgG, and human serum proteins adsorption isotherms

Single component adsorption isotherms at 25 °C for IgG and HSA are shown in Fig. 5b and c. An excellent fit with experimental data was achieved using Langmuir model (Table 4). Adsorption favored HSA ($K_d = 4.97 \times 10^{-6}$ mol/L) when compared to IgG ($K_d = 2.11 \times 10^{-5}$ mol/L). Also, the IgG binding capacity (38.52 mg/mL of gel) was five times lower than the value for HSA (191.70 mg/mL of gel). The lower capacity for IgG is in accordance with the electrostatic mode of interaction as discussed in Section 3.1. The maximum capacity for the HSA obtained for TREN-agarose was approximately two times higher than the maximum capacity for BSA and HSA onto two different adsorbents: 89.5 ± 2.3 mg BSA/mL of DEAE-cellulose [37] and 93.3 mg HSA/mL of histidine grafted aminohexyl-Sepharose [38].

Table 4
Langmuir parameters for the adsorption of HSA, IgG and human serum proteins onto TREN-agarose gel.

Proteins	Q_m (mg/mL)	K_d (mol/L)	R^2
HSA	191.70 ± 6.18	4.97 ± 0.87 × 10^{-6}	0.98
IgG	38.25 ± 1.24	2.11 ± 0.26 × 10^{-5}	0.99
Human serum	66.63 ± 2.34	–	0.97

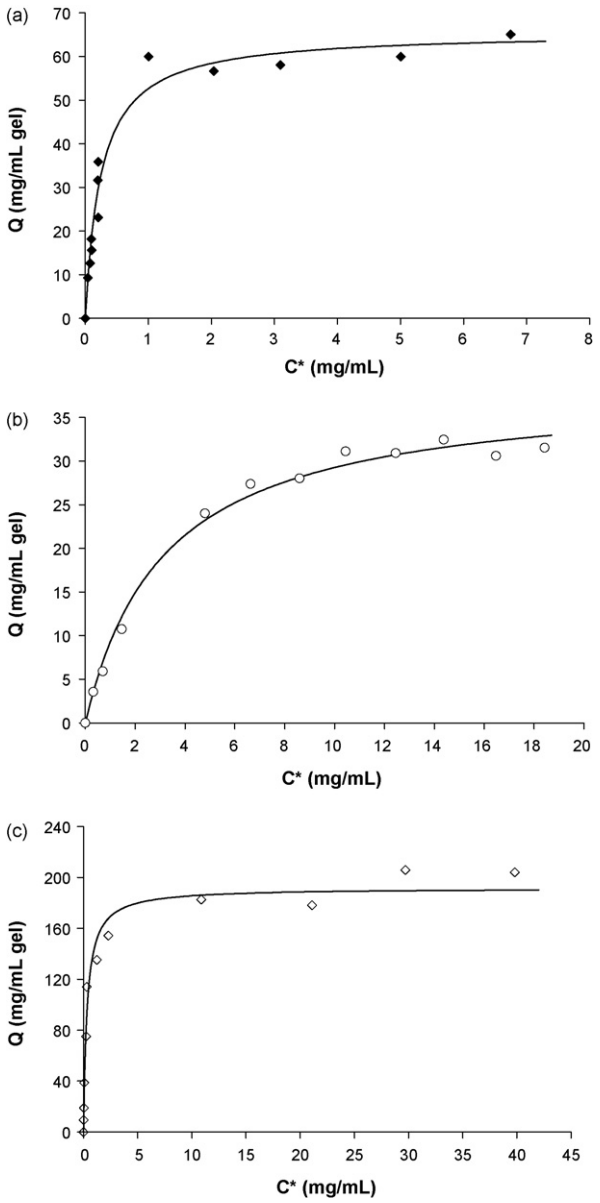

Fig. 5. Experimental adsorption isotherms (symbols) for (a) human serum, (b) IgG and (c) HSA on TREN-agarose gel with Mes buffer, pH 6.5, at 25 °C. The solid lines correspond to fitting (non-linear regression) of experimental data in accordance with Langmuir model.

The isotherm at 25 °C for human serum and the related Langmuir parameters are presented in Fig. 5a and Table 4, respectively. There was a decrease in the maximum binding capacity of protein when compared with the value for HSA (66.63 and 191.70 mg/mL of gel, respectively). Although we did not investigate why this difference occurred, but we can elaborate a few hypothesis about possible reasons. The serum has a higher conductivity and slightly higher pH than its solutions. The proteins in the serum may adsorb with negative cooperativity towards HSA (repulsion due lateral interactions) or may cause steric hindrance, decreasing HSA adsorption. Also protein-protein interaction may form complexes that do not adsorb to the gel.

A true dissociation constant (K_d) could not be determined since adsorption isotherm was constructed using human serum protein, a complex mixture.

4. Conclusion

We have demonstrated that negative chromatography using the nonbiological ligand TREN immobilized onto agarose gel beads is a simple and efficient method for the purification of IgG from human serum in a simple step. The adsorption of IgG and serum proteins was influenced by the nature of the buffer and pH. The TREN-agarose adsorbent had a capacity and selectivity for negative IgG purification similar to that of histidine grafted aminohexyl-Sepharose. IgG recovery from human serum could be achieved under mild conditions of pH, low ionic strength, and room temperature. From a large-scale process point of view, negative chromatography using TREN-agarose gel can be used as the basis for a new, more robust (nonbiological ligand) and lower cost process.

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