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Purification of human IgG by negative chromatography on ω-aminohexyl-agarose

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ABSTRACT

The ω-aminohexyl diamine immobilized as ligand on CNBr- and bisoxirane-activated agarose gel was evaluated for the purification of human immunoglobulin G (IgG) from serum and plasma by negative affinity chromatography. The effects of matrix activation, buffer system, and feedstream on recovery and purity of IgG were studied. A one-step purification process using Hepes buffer at pH 6.8 allowed a similar recovery (69–76%) of the loaded IgG in the nonretained fractions for both matrices, but the purity was higher for epoxy-activated gel (electrophoretically homogeneous protein with a 6.5-fold purification). The IgG and human serum albumin (HSA) adsorption equilibrium studies showed that the adsorption isotherms of IgG and HSA obeyed the Langmuir–Freundlich and Langmuir models, respectively. The binding capacity of HSA was high (210.4 mg mL^{-1} of gel) and a positive cooperativity was observed for IgG binding. These results indicate that immobilizing ω-aminohexyl using bisoxirane as coupling agent is a useful strategy for rapid purification of IgG from human serum and plasma.

1. Introduction

Polyamines are polycations that are able to interact with proteins, nucleic acids, and phospholipids [1–5]. Due to their structure, polyamines interact with negatively charged molecules by electrostatic binding. Despite their polycationic nature, polyamines also have the potential for hydrophobic interactions, depending on the length of alkyl chain. Numerous studies have indicated that polyamines are selective and efficient immobilized ligands for purification of proteins with affinity for amino groups [1].

Different polyamine-grafted chromatographic matrices have been used as affinity adsorbents for purification of many proteins. Like pseudobiospecific ligands, polyamines are low cost and have high stability, capacity, simplicity, and selectivity [1]. They can be easily immobilized after derivatization of different matrices with various activating agents such as cyanogen bromide (CNBr), epichrorohydrin, bisoxiranes, or divinylsulfones.

Diamines such as 1,5-diaminopentane (aminopentyl) and 1,6-diaminohexane (aminohexyl) have been immobilized as ligands on CNBr- or divinylsulfonyl-activated agarose for purification of amine oxidases [6,7]. As lactoferrin (a protein with a high isoelectric point) binds to diamines by affinity interactions, it has been purified using aminohexyl immobilized on divinylsulfone-activated agarose [8]. Plasminogen binds to lysine-Sepharose and can be purified in one step using an α-carboxy-aminoalkyl gel [9]. More complex polyamines such as spermidine, spermine, and TREN (Tris(2-aminoethyl)amine)-grafted chromatographic matrices have been used for the purification of spermine synthase, ryanodine receptor, and human immunoglobulin G (IgG), respectively [10–12].

TREN, a polyamine with four amino groups (four nitrogen atoms, three of which are primary in nature and the fourth one is tertiary), adsorbed human serum proteins (probably by electrostatic interactions) and human IgG could be purified by negative chromatography. In the negative chromatographic mode, the impurities or contaminants are adsorbed by the adsorbent and the product is collected in the flowthrough and washing fractions [12–14].

Although immobilized polyamines have been used successfully for purification of different proteins, the effect of amino residues content, the nature of the amino group (primary, secondary, or tertiary amino group), and the length of the alkyl chain in protein purification are not yet known.

Thus, the aim of this work was to study whether a simpler polyamine such as an aliphatic amine would be better for use in human IgG purification than the more complex polyamines (such as TREN) used in the reported studies. This work evaluated the feasibility of using the ligand aminohexyl (1,6-diaminohexane) immobilized on agarose gel for the purification of IgG from human serum or plasma solutions by negative chromatography. The effects of the spacer arm and operating conditions (type of feed, pH, buffer system, concentration of IgG in feed solutions) on the capacity and selectivity of the system were also investigated. The breakthrough curve and dynamic capacity of serum proteins were determined to obtain data useful for the development of large-scale

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processes for purification of human IgG in the negative chromatography mode. IgG and the HSA binding were also studied and the adsorption isotherms were analyzed with the Langmuir and Langmuir–Freundlich models. Parameters pertinent to the adsorption processes such as the apparent dissociation constant (K_d), the maximum binding capacity (Q_m), and the cooperativity were analyzed and discussed.

2. Experimental

2.1. Materials

The ω-aminohexyl immobilized on CNBr and on epoxy-activated agarose (cross-linked 4% beaded), referred to in this work as ω-aminohexyl-agarose and ω-aminohexyl-bisoxirane-agarose, respectively, were purchased from Sigma (USA).

For SDS-PAGE analysis, acrylamide, bis-acrylamide, sodium dodecyl sulfate (SDS), and dithiotrietol were purchased from Bio-Rad (USA). High molecular mass markers for SDS-PAGE (myosine, 212 kDa; α2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa) were purchased from GE Healthcare (USA). Coomassie Brilliant Blue and Morpholinoethane sulfonic acid (Mes) were purchased from Merck (Germany). Morpholinopropane sulfonic acid (Mops), Hydroxyethylpiperazine ethanesulfonic acid (Hepes), crystalline bovine serum albumin (BSA), and prepurified human serum albumin (HSA, 99% purity) were provided by Sigma (USA). Prepurified human immunoglobulin G (with 98.3% IgG according to nephelometric analysis of IgG, IgM, IgA, albumin (HSA), and transferrin (Trf) 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. Human serum and plasma samples

Blood samples from a healthy donor were collected in Vacuette® serum tubes or in Vacuette® plasma tubes (Greiner BioOne, Austria). The serum tubes were kept at room temperature for about 2 h in order to allow agglutination to obtain serum. The samples collected in serum and plasma tubes were centrifuged at 4 °C for 5 min at 3000 rpm 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 or plasma samples with the proper adsorption buffer.

2.3. Chromatographic experiments

All chromatographic experiments were carried out using an automated chromatography system (ÄKTA Prime Plus, GE Healthcare, USA) at 25 °C at a flow rate of 0.5 mL min^{-1} (linear flow rate of 38.2 cm h^{-1}). For studies concerning the influence of the buffer on human serum and human plasma protein adsorption, the following loading buffers at 25 mmol L^{-1} covering a pH range from 6.5 to 8.2 within their respective buffering ranges were used: Mops, Mes, and Hepes.

The ω-aminohexyl-agarose and ω-aminohexyl-bisoxirane-agarose gels were 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 3.0 mL. The column was equilibrated with equilibration buffer (25 mmol L^{-1} Mops, Mes, or Hepes buffer). The human serum or plasma was diluted 20 times with the equilibration buffer and loaded into the column. After protein injection, the column was washed with equilibration buffer until protein was no longer detected in the column out-stream by absorption at 280 nm. Elution was performed with the loading buffer containing 1.0 mol L^{-1} NaCl.

During the wash and elution steps, absorbance at 280 nm was monitored and fractions of 1.0 and 2.0 mL, respectively, were collected. The Bradford method [15], 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 mmol L^{-1} NaOH, followed by water, and finally by the loading buffer to restore it to its initial conditions (regeneration) for carrying out the next experiment.

2.4. Adsorption breakthrough curves

These experiments were carried out at 25 °C with an automated chromatography system (ÄKTA Prime Plus, GE Healthcare, USA). After equilibration of ω-aminohexyl-bisoxirane-agarose with the loading buffer (25 mmol L^{-1} Hepes buffer at pH 6.8), 106.0 mL of human serum diluted 20 times in Hepes buffer at pH 6.8 (359.58 mg of total serum protein) 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 mmol L^{-1} Hepes buffer at pH 6.8, containing 1.0 mol L^{-1} 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 mmol L^{-1} NaOH, followed by water and the loading buffer.

Protein concentrations in the retained and nonretained fractions were determined by the Bradford method [15] and nephelometric analysis and analyzed by SDS-PAGE under nonreducing conditions. The ratio of the outlet total protein concentration (C) to that in the feedstream (C_0) was plotted in a breakthrough curve as a function of the volume of protein solution throughput.

2.5. Protein adsorption studies

Experiments to determine the adsorption isotherms of HSA and human IgG on ω-aminohexyl-bisoxirane-agarose at 25 °C were carried out (in duplicates) in Eppendorf tubes (as stirred tanks) with 0.05 mL of gel. The gel had been previously equilibrated with degassed 25 mmol L^{-1} Hepes buffer at pH 6.8 and the aliquots of 1.0 mL HSA or IgG solutions were added to the tubes. The initial protein concentrations of HSA and IgG were in the range of 0.5–50.0 mg mL^{-1} and 0.5–36.0 mg mL^{-1}, respectively. The tubes were agitated for 3 h to allow equilibrium to be established. After this, the protein equilibrium concentration in the liquid phase (C) was quantified. The concentration of protein was measured based on absorbance at 280 nm (UV–vis spectrophotometer, Beckman DU 650, USA). The difference in unbound protein concentrations between the experiments did not exceed on average 2.0% and 0.5% for IgG and HSA, respectively. The mass of protein adsorbed per volume of gel (mg mL^{-1}), Q, was calculated as the difference between the amount of protein added and that remaining in the liquid phase after equilibrium divided by the volume of the adsorbent. Plotting Q as a function of C yielded the equilibrium isotherm. The Langmuir (Eq. (1)) and Langmuir–Freundlich (Eq. (2)) isotherm models [16,17] were used to fit the data:

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

$$Q = \frac{Q_m C^n}{K_{dLF} + C^n} \quad (2)$$

where Q_m is the maximum protein binding capacity; K_d is the dissociation constant, which represents the affinity between protein and adsorbent; K_{dLF} is the apparent dissociation constant that includes contributions from ligand binding to monomer, monomer–dimer, and more highly associated forms of the protein; and n is the Langmuir–Freundlich coefficient. By analogy with protein–multiple ligand interactions it has been suggested that Eq. (2) works well for cooperativity model adsorption [17,18]. For purely independent noninteracting sites, $n = 1$. Negative cooperativity in the binding process is indicated when $0 < n < 1$, while for positive cooperativity of the protein binding sites, $n > 1$. The value of n can thus be employed as an empirical coefficient, indicating the type and the extent of cooperativity present in the binding interaction [17].

The parameters of the Langmuir and Langmuir–Freundlich models were fitted to the experimental data employing the iterative fitting method of Levenberg–Marquardt, using Statistica® (Statsoft, USA).

2.6. Analytical methods

2.6.1. Protein quantification

The total protein concentration of the fractions collected in the chromatographic runs was quantified by the Bradford method [15], using bovine serum albumin (BSA) as reference protein. In experiments containing prepurified IgG and prepurified HSA, the protein concentrations were determined by measuring the absorbance at 280 nm.

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

The chromatographic fractions were analyzed by SDS-PAGE under nonreducing conditions [19] using a Mini-Protean III system (BioRad, USA). The separation was carried out at 180 V in 7.5% separation gels with a 4% stacking gel. Protein bands were developed by silver staining [20]. 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.

2.6.3. Analysis of HSA, IgA, IgG, IgM, and Trf

The concentrations of IgA, IgG, IgM, HSA, and Trf in the fractions collected in the chromatographic experiments were determined nephelometrically using an Array Protein System (Beckman, USA), according to the method provided by the manufacturer.

3. Results and discussion

3.1. Effect of pH and matrix activation on IgG purification

3.1.1. Effect of pH on IgG recovery in nonretained fractions and the selectivity of the adsorbent

Three variables were used to analyze the efficiency of amino-adsorbent: percentage of unbound IgG, based on the percentage of IgG fed to the adsorbent that was not retained; percentage of total protein eluted, based on the percentage of whole serum protein retained that was eluted (adsorption capacity of total serum protein); and the purity of unbound IgG (selectivity of adsorbent).

The pH of the mobile phase had a significant impact on the IgG purification by negative chromatography. The pH affected the charged state of amino acid residues and other groups that may be involved in the binding interaction, and a net charge of serum protein molecules changed in the range of pH studied [21]. The range of pH considered (6.5–8.2) is within the pH values commonly employed in chromatography by maintaining the protein in an environment similar to that of biological fluids.

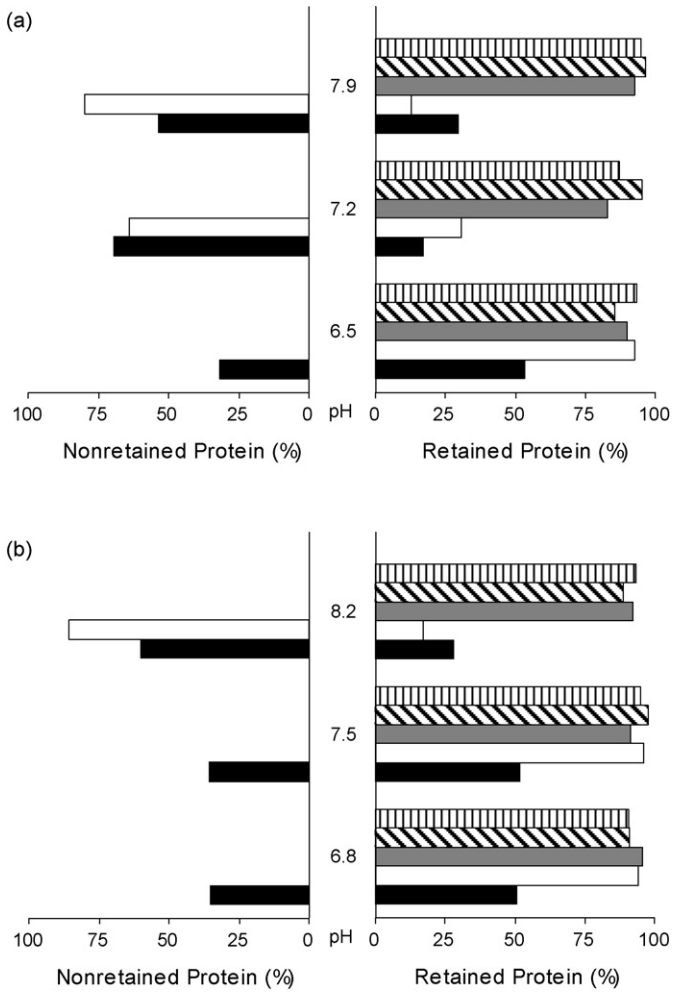

Fig. 1. Effect of buffer system ((a) Mops, (b) Hepes) and pH on retention and nonretention of human (■)IgG, (□) Trf, (▩) HSA, (▨) IgA, and (▥) IgM by ω-aminohexyl-agarose adsorbent. The retained and nonretained fractions were analyzed by nephelometry.

Adsorption of protein using ω-aminohexyl-agarose (CNBr-activated gel) was investigated loading 50 μL of untreated human serum diluted 20 times (1.0 mL of serum solution) with three different Good's buffers [22] (Mes, Mops, and Hepes), covering a pH range of 6.5–8.2 in order to determine the condition most favorable for whole serum protein adsorption. Retained and nonretained fractions were analyzed by the Bradford method [15], nephelometry, and SDS-PAGE.

The capture of serum proteins and purification of IgG varied in ω-aminohexyl-agarose, depending on the buffer system. In Mes buffer, the serum proteins were partially captured and IgG obtained in nonretained fractions was contaminated with several serum proteins (data not shown). The effect of the pH of Mops and Hepes adsorption buffers on the binding capacity for serum proteins is shown in Fig. 1. The results were evaluated in terms of selectivity and IgG recovery in the flowthrough and washing streams. The percentage of nonretained IgG increased with increasing pH; however, at pH higher than 7.2–7.5, the percentage of nonretained Trf drastically increased. Approximately the same percentage of IgG was nonretained in Mops buffer at pH 6.5 (31.6%) as in Hepes at pH 6.8 (35.1%). However, the purities of IgG (based on the ratio of IgG determined by nephelometry and total protein determined with the Bradford method [15]) obtained for Mops and Hepes buffers were 86.7 and 95.6%, respectively. Therefore, the optimum combination of yield (35.1%) and purity (95%) of IgG recovered

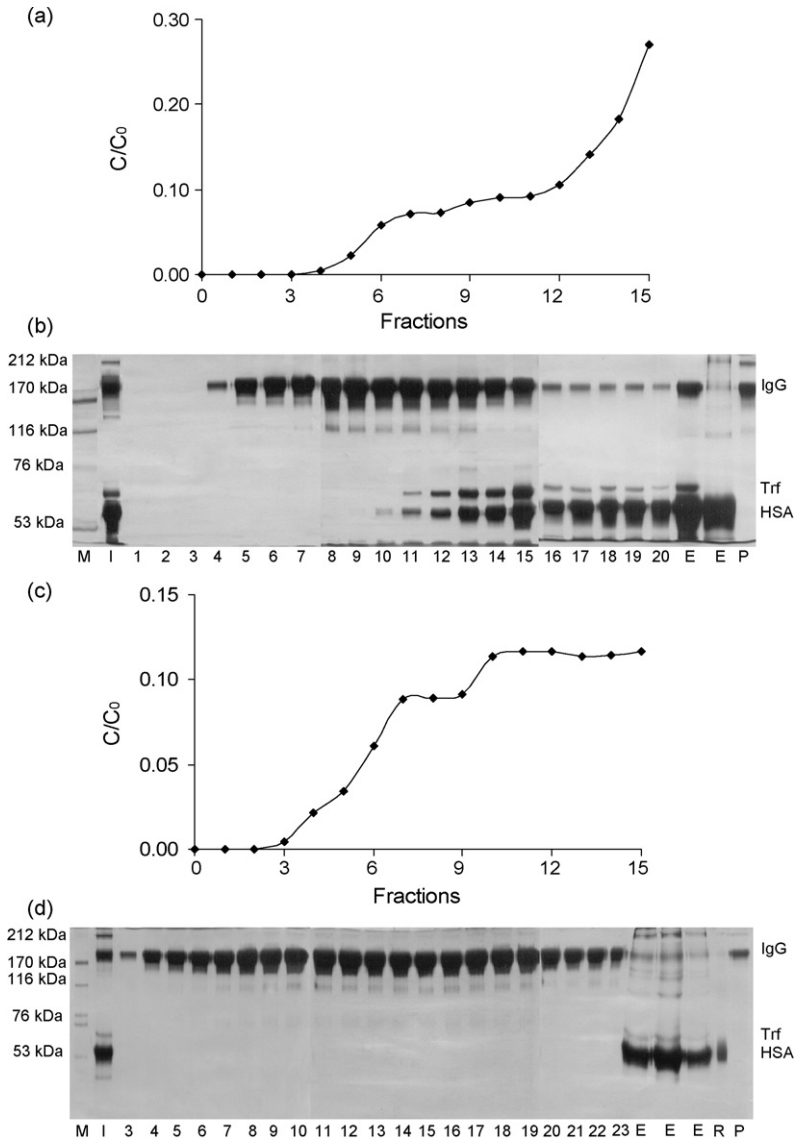

Fig. 2. Effect of the activating agent on purification of IgG from human serum diluted 20 times in Hepes buffer at pH 6.8 by negative chromatography. (a) ω-Aminohexyl-agarose (CNBr-activated gel); (c) ω-aminohexyl-bisoxirane-agarose (epoxy-activated gel). (b) and (d) Nonreducing SDS-PAGE analysis of fractions from chromatography on ω-aminohexyl-agarose and ω-aminohexyl-bisoxirane-agarose gels, respectively: M, molecular mass protein marker; I, injected human serum solution; numbers in SDS-PAGE correspond to the nonretained fractions indicated in chromatograms; E, retained fractions (eluted with the adsorption buffer containing 1.0 mol L^{-1} NaCl); R, pool of regenerated fractions; P, human IgG standard (Aventis Behring).

by negative chromatography was obtained with Hepes buffer at pH 6.8.

3.1.2. *Effect of matrix activation on IgG purification*

The matrix activation and spacer arm introduced into the adsorbent also play an important role in the efficiency of IgG purification by negative chromatography, due to the large molecules present in human serum.

Untreated human serum solution (750 μL of human serum diluted 20 times in Hepes buffer at pH 6.8, conductivity of 777 μS cm^{-1}) was loaded onto ω-aminohexyl-agarose (CNBr-activated gel, spacer arm of 1 atom) and ω-aminohexyl-bisoxirane-agarose (epoxy-activated gel, spacer arm of 12 atoms) in order to investigate the effect of spacer arm on IgG purification. The activation of agarose gel with bisoxirane incorporated a longer spacer arm into the adsorbent. Thus, the amino groups were sufficiently far from the support surface, thus decreasing the steric hindrance effects between the ligand and the agarose beads, allowing the highest yield and selectivity of nonretained IgG (Fig. 2 and Table 1).

The immobilization of aminohexyl via the short spacer CNBr (7.4 μmol of aminohexyl mL^{-1} of gel, Sigma–Aldrich certificate of analysis) resulted in a ligand density similar to that epoxy-activated gel (7.0 μmol of aminohexyl mL^{-1} of gel, Sigma–Aldrich certificate of analysis). For CNBr-activated gel, only 30% of the loaded IgG was obtained with high purity in nonretained fractions (fractions 4–9, Fig. 2), in contrast with epoxy-activated gel, in which 76% of IgG was recovery in nonretained fractions with high purity (Fig. 2 and Table 1). The combination of flowthrough and washing fractions resulted in an IgG purity of 111% (based on total protein and IgG, IgA, IgM, HSA, and Trf determined with the Bradford method [15] and nephelometric analysis, respectively) for epoxy-activated gel, while for CNBr-activated gel the purity was 30%. The purity of IgG in nonretained fractions for epoxy-activated gel was higher than 100% because the Bradford method [15] shows a variation in response to different proteins (low sensitivity to the IgG proteins of the samples) [23,24].

For ω-aminohexyl-bisoxirane-agarose, the IgG purification factor was 6.5 (based on a combination of flowthrough and washing

Table 1
Effect of matrix activation on purification of IgG from human serum.

Fractions	ω-Aminohexyl-agarose[a]							ω-Aminohexyl-bisoxirane-agarose[b]						
	TP[d] (mg)	IgG (mg)	IgA (mg)	IgM (mg)	HSA (mg)	Trf (mg)	Purity (%)	PF[e]	TP[d] (mg)	IgG (mg)	IgA (mg)	IgM (mg)	HSA (mg)	Trf (mg)	Purity (%)	PF[e]
Initial solution[c]	50.50[f]	7.89	1.95	0.66	39.15	2.00	15.6	1.0	49.40[f]	8.50	1.03	1.09	32.25	1.69	17.2	1.0
Flowthrough	10.55	3.70	nd[g]	nd	5.95	0.96	35.1	2.3	3.36	4.00	nd	nd	nd	nd	119.0	6.9
Washing	7.90	1.77	nd	nd	4.95	0.80	22.4	1.4	2.38	2.46	nd	nd	nd	nd	103.3	6.0
Elution	29.50	1.13	1.74	0.62	26.30	0.20	3.8	0.3	40.83	1.78	0.98	1.04	31.39	1.67	4.4	0.3
Regeneration	0.10	–	–	–	–	–	–	–	0.27	–	–	–	–	–	–	–
Total recovery	48.05	6.60	1.74	0.62	37.20	1.96	–	–	46.84	8.24	0.98	1.04	31.39	1.67	–	–

[a] CNBr-activated gel (spacer arm of 1 atom).
[b] Epoxy-activated gel (spacer arm of 12 atoms).
[c] Human serum diluted 20 times in Hepes buffer at pH 6.8 (15.0 mL).
[d] TP: total protein.
[e] Purification factor.
[f] Conductivity of 777 μS cm^{-1}.
[g] Values lower than the detectable range of Array Protein System.

fractions) and the purity was higher than that of the commercial IgG product (based on SDS-PAGE analysis).

The higher selectivity of ω-aminohexyl-bisoxirane-agarose could be associated with the higher adsorption capacity for serum proteins (13.61 mg of total serum protein mL^{-1} of adsorbent). These results suggest that the long spacer arm (12 atoms) had a positive contribution in serum protein binding.

A similar observation was also reported in the literature. For example, using a IAV membrane matrix, Suen and Tsai indicated that a spacer arm length of 12 atoms was effective for the elimination of steric hindrance of ligand (Cibacron Blue 3GA) accessibility, offering the best lysozyme adsorption capacity [25]. The effect of spacer arm size on the pseudoaffinity adsorbent (L-arginine ligand attached to poly(glycidiyl methacrylate/methyl methacrylate/ethylene glycol dimethacrylate—poly(GMA/MMA/EGDMA)) for IgG adsorption was investigated by Bayramoglu et al. These authors observed that the maximum adsorption on the aminohexyl spacer arm attached beads was higher than that on the pseudoaffinity beads without a spacer arm [26].

The optimum combination of yield and purity of IgG recovered from human serum with ω-aminohexyl-bisoxirane-agarose adsorbent was similar to the results presented for TREN ligand immobilized on agarose reported by Bresolin et al. [12]. These authors used the polyamine TREN as ligand to purify IgG from human serum by negative chromatography, obtaining 68% recovery of IgG in nonretained fractions with a purity of 90–95% [12]. The highlight of the results achieved in the present work and by Bresolin et al. [12] is that in unlike with other affinity chromatographic methods (using protein A and G as affinity ligand), high-purity nonretained IgG was recovered with under mild conditions.

3.2. Dynamic capacity of ω-aminohexyl-bisoxirane-agarose

In order to determine the protein adsorption dynamic capacity of ω-aminohexyl-bisoxirane-agarose, the column was overloaded with 106.0 mL of human serum diluted 20 times in Hepes buffer at pH 6.8 (359.58 mg of total serum protein) at a flow rate of 0.5 mL min^{-1} so that the adsorbent would be saturated. Fig. 3 displays the breakthrough curve quantified by the Bradford method [15] and the SDS-PAGE of retained and nonretained fractions. The binding results for IgA, IgG, IgM, HSA, Trf, and total protein and unbound IgG are summarized in Table 2. The dynamic capacity of ω-aminohexyl-bisoxirane-agarose under saturating conditions was calculated as 49.77 mg of total serum protein mL^{-1} of adsorbent.

Defining the breakthrough point as the fraction where HSA and other serum protein are detected by SDS-PAGE and nephelometric analysis, a volume of 48.0 mL of human serum solution (16.0 mL of human serum solution mL^{-1} of adsorbent) was loaded prior to breakthrough, corresponding to 21.13 mg of unbound IgG (7.04 mg of IgG mL^{-1} of adsorbent). The SDS-PAGE result shows that the nonretained fractions 4–24 are homogeneous and their purity is higher than that of the commercial IgG product (based on SDS-PAGE analysis). These flowthrough fractions correspond to 29.1% of nonretained highly pure IgG, based on the percent of IgG fed to the adsorbent that was not retained. The protein impurities that were observed in flowthrough fractions after 48.0 mL could be removed by rechromatographing the nonretained fractions using ω-aminohexyl-bisoxirane-agarose (data not shown).

Comparing the polyamine TREN-grafted agarose used as a negative adsorbent for purification of IgG from human serum [12] and the diamine aminohexyl-grafted agarose, a similar volume (12 and 16 mL mL^{-1} of adsorbent for TREN agarose and ω-aminohexyl-bisoxirane-agarose, respectively) of human serum solution was loaded prior to breakthrough. Both adsorbents had similar amounts of unbound IgG in flowthrough (7.0 mg of IgG mL^{-1} of adsorbent), but the purity was significantly better for ω-

Table 2
Nephelometric analysis of IgA, IgG, IgM, HSA, and Trf after chromatographic purification of human serum on ω-aminohexyl-bisoxirane-agarose gel.

Fractions	TP[b] (mg)	IgG (mg)	IgA (mg)	IgM (mg)	HSA (mg)	Trf (mg)	Purity (%)	PF[c]
Initial solution[a]	359.58[d]	72.5	22.40	9.68	213.1	18.70	20.2	1.0
Fractions 4–24	15.38	21.13	nd[e]	nd	nd	nd	137.4	6.8
Fractions 25–56	194.21	42.50	nd	nd	109.0	17.90	21.9	1.1
Elution	149.30	6.68	20.51	8.96	128.0	1.53	4.5	0.2
Regeneration	0.52	–	–	–	–	–	–	–
Total recovery	359.41	70.31	20.51	8.96	237.0	19.43	–	–

[a] Human serum diluted 20 times in Hepes buffer at pH 6.8 (106.0 mL).
[b] TP: total protein.
[c] Purification factor.
[d] Conductivity of 777 μS cm^{-1}.
[e] Values lower than the detectable range of Array Protein System.

aminohexyl-bisoxirane-agarose (based on IgG, IgA, IgM, HSA, and Trf determined by nephelometric methods and SDS-PAGE analysis).

3.3. IgG interactions with amino ligand

The mechanism of the interaction between the ω-aminohexyl-bisoxirane-agarose and human serum proteins is not yet known, but may be a result of a combination of electrostatic interactions and an affinity of serum proteins for primary amino groups.

Electrostatic interactions were probably predominant between human serum proteins and ω-aminohexyl-bisoxirane-agarose, since elution was possible with the addition of salt (NaCl). In fact, aminoalkyl matrices containing primary amino groups such as aminohexyl are weak anion exchangers [1], which are protonated at pH 6.8. Even when the overall net charge of protein molecules is of the same sign as that of the adsorbent, the adsorption of molecules in ion exchangers can occur due to the presence of local clusters of charged groups with the opposite sign [27]. However, many proteins have an affinity for amino groups [1]; therefore interactions other than electrostatic can be involved in the retention of serum protein on ω-aminohexyl-bisoxirane-agarose.

Concerning IgG molecules, the protein adsorption seemed to be governed by electrostatic force. Isoelectrofocusing of prepurified human IgG chromatography fractions showed the presence of molecules with high pI in the range of pH 7.5–9.0 in nonretained fractions, while the IgG used for the experiments had pI ranging from 5.5 to 9.0. Therefore, when pH < pI, the amino acid residues such as histidine, lysine, and arginine are found to be protonated, and hence their ability to interact ionically with amino groups of the adsorbent was reduced. The eluted chromatographic fractions analyzed contained IgG with pI from 5.5 to 7.5 (electrophoresis not shown).

Roch et al. [28] reported the existence of a specific interaction of human IgG antibodies present in human sera with polyamines (spermine and putrescine). The binding studies developed by Bouillé et al. [2] with an antispermine monoclonal antibody revealed that aspartic acid and tyrosine within the antibody combining site were important contact residues for electrostatic interactions and hydrogen bonding with the tetra positively charged spermine molecule. Therefore, the results obtained for human IgG adsorption on aminohexyl are in accordance with the electrostatic mode of interaction proposed by Bouillé et al. [2].

3.4. Effect of feedstream on IgG purification

Human plasma is a highly complex biological material, containing hundreds of proteins with very different molecular masses, concentrations, pIs, and physiological functions [29]. Apart from other serum proteins, whole human plasma contains fibrinogen, coagulation factors, and other plasma proteins as significant contaminants. Thus, the challenges facing the purification of IgG from human serum are different from those facing the purification of IgG from human plasma.

Significant protein precipitation was observed when human plasma was diluted with 25 mmol L^{-1} Hepes at pH below than 7.5. Therefore, the chromatographic experiments were carried out at pH 8.2 on ω-aminohexyl-bisoxirane-agarose for human plasma diluted 20 times with 25 mmol L^{-1} Hepes buffer (conductivity of 1453 μS cm^{-1}, pH 8.19). Fig. 4 and Table 3 show a summary of the retained and nonretained proteins.

The binding capacity of the gel and the purity of IgG obtained in the flowthrough and washing fractions were similar to those in experiments carried out with serum and plasma solutions (Tables 1 and 3, respectively). However, IgG recovery in nonretained fractions was higher when human serum was fed into the

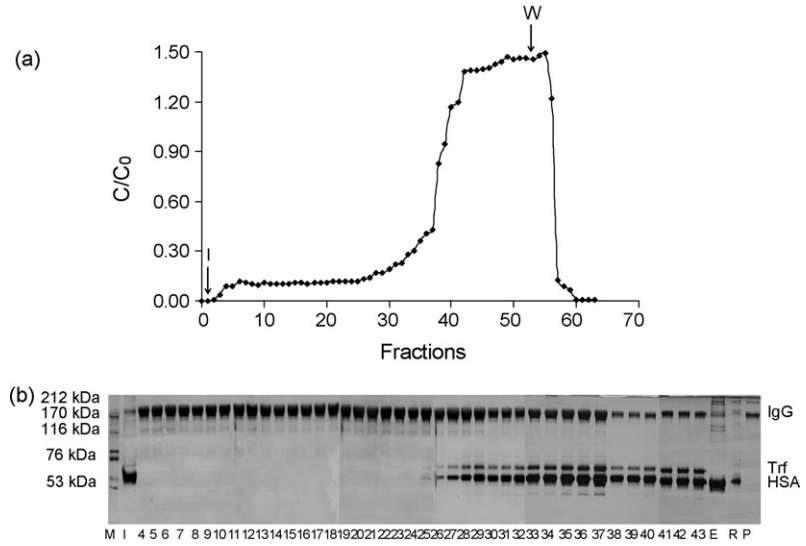

Fig. 3. (a) Breakthrough curve of human serum solution diluted 20 times in Hepes buffer at pH 6.8 for ω-aminohexyl-bisoxirane-agarose gel. I: injection: 106.0 mL at 3.39 mg of total protein mL^{-1}; W: washing. (b) SDS-PAGE analysis under nonreducing conditions of fractions of breakthrough curve: M, molecular mass protein marker; 4–43, nonretained fractions; E, pool of retained fractions (eluted with the adsorption buffer containing 1.0 mol L^{-1} NaCl); R, pool of regenerated fractions; P, human IgG standard (Aventis Behring).

column (76% and 64% for experiments carried out with serum and plasma solutions, respectively).

Changing the plasma feedstream dilution from 20 times to 10 times and the adsorption buffer from Hepes buffer at pH 8.2 to Mops buffer at pH 7.9 had an insignificant effect on IgG purification by negative chromatography (Fig. 5 and Table 4). Comparing these results with those of the experiments carried out with plasma diluted 20 times in Hepes buffer at pH 8.2, the total plasma protein retention (77% and 78% for plasma diluted 20 times and 10 times, respectively) on ω-aminohexyl-bisoxirane-agarose was not affected by the conductivity and pH of the medium (conductivity of 1453 μS cm^{-1}, pH 8.19 and 1759 μS cm^{-1}, pH 7.83, respectively). Furthermore high-purity IgG was obtained in nonretained fractions (100.9% IgG purity) when plasma was diluted 10 times; however, the IgG recovery in nonretained fractions was lower (55%).

The resulting purity and recovery on ω-aminohexyl-bisoxirane-agarose obtained in this study by negative chromatography were comparable to those in data from the literature on histidine-grafted aminohexyl-Sepharose [14]. These authors obtained 6.46 mg of electrophoretically pure IgG (corresponding to 72.6% of the injected IgG) in nonretained fractions when 1.0 mL of human plasma solution (diluted 20 times in Mops buffer at pH 7.2) was fed into the column.

As human plasma is the source material for the production of pharmaceutical fractionated products (hemoderivatives), it is also very important to maximize the recovery of adsorbed plasma

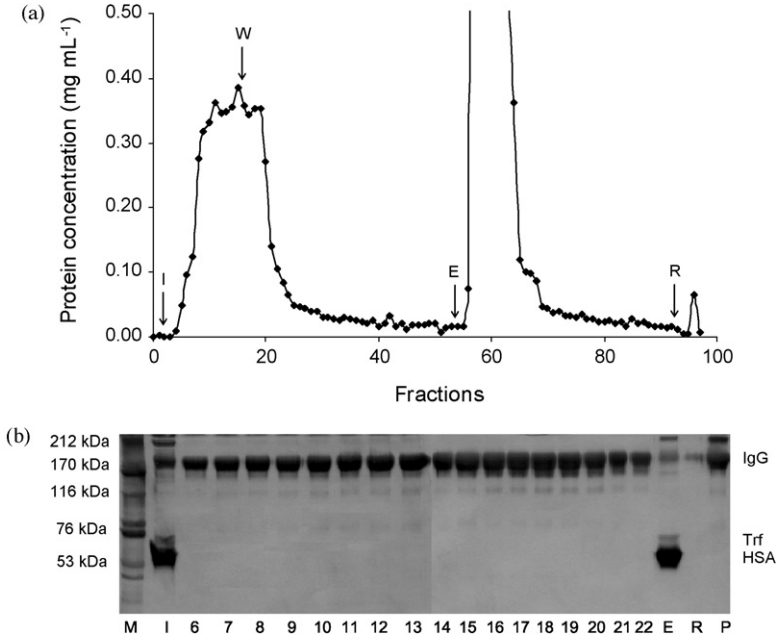

Fig. 4. (a) Chromatogram of human plasma diluted 20 times in Hepes buffer at pH 8.2 on ω-aminohexyl-bisoxirane-agarose gel. Flow rate: 0.5 mL min^{-1}; I: injection, 15.0 mL at 3.29 mg of total protein mL^{-1}; W: washing, Hepes buffer at pH 8.2; E: elution, Hepes buffer at pH 8.2 containing 1.0 mol L^{-1} of NaCl; R: regeneration, 25 mmol L^{-1} of NaOH. (b) SDS-PAGE analysis under nonreducing conditions of fractions of breakthrough curve: M, molecular mass protein marker; 6–22, nonretained fractions; E, pool of retained fractions (eluted with the adsorption buffer containing 1.0 mol L^{-1} NaCl); R, pool of regenerated fractions; P, human IgG standard (Aventis Behring).

Table 3
Purification of IgG from human plasma diluted 20 times in Hepes buffer at pH 8.2 on ω-aminohexyl-bisoxirane-agarose gel.

Fractions	TP[b] (mg)	IgG (mg)	IgA (mg)	IgM (mg)	HSA (mg)	Trf (mg)	Purity (%)	PF[c]
Initial solution[a]	49.37[d]	8.48	0.90	1.74	29.70	1.82	17.2	1.0
Flowthrough	2.90	3.07	nd[e]	nd	nd	nd	105.9	6.2
Washing	1.91	2.39	nd	nd	nd	nd	125.1	7.3
Elution	37.90	2.57	0.86	1.63	27.50	1.75	6.8	0.4
Regeneration	0.16	–	–	–	–	–	–	–
Total recovery	42.87	8.03	0.86	1.63	27.50	1.75	–	–

[a] Human plasma diluted 20 times in Hepes buffer at pH 8.2 (15.0 mL).
[b] TP: total protein.
[c] Purification factor.
[d] Conductivity of 1453 μS cm^{-1}, pH 8.19.
[e] Values lower than the detectable range of Array Protein System.

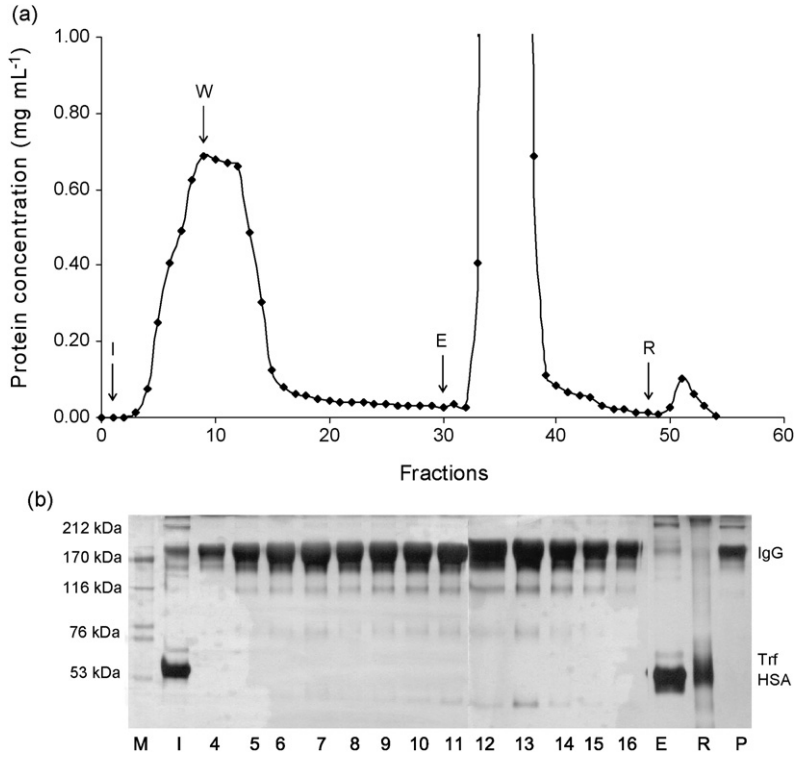

Fig. 5. (a) Chromatogram of human plasma diluted 10 times in Mops buffer on ω-aminohexyl-bisoxirane-agarose gel. Flow rate: 0.5 mL min^{-1}; I: injection, 8.0 mL at 6.46 of total protein mL^{-1}; W: washing, Mops buffer at pH 7.9; E: elution, Hepes buffer at pH 8.2 containing 1.0 mol L^{-1} of NaCl; R: regeneration, 25 mmol L^{-1} of NaOH. (b) SDS-PAGE analysis under nonreducing conditions of fractions of breakthrough curve: M, molecular mass protein marker; 4–16, nonretained fractions; E, pool of retained fractions (eluted with the adsorption buffer containing 1.0 mol L^{-1} NaCl); R, pool of regenerated fractions; P, human IgG standard (Aventis Behring).

proteins. The results obtained showed that the desorption efficiency produced a high recovery of the total plasma protein loaded into the column (up to 87%), showing that additional downstream methods may be used for fractionation of the eluted proteins.

3.5. IgG and HSA adsorption isotherms

The equilibrium adsorption data on single protein IgG and HSA on ω-aminohexyl-bisoxirane-agarose were obtained at 25 °C from batch experiments using Hepes buffer at pH 6.8. The experimen-

Table 4
Purification of IgG from human plasma diluted 10 times in Mops buffer at pH 7.9 on ω-aminohexyl-bisoxirane-agarose gel.

Fractions	TP[b] (mg)	IgG (mg)	IgA (mg)	IgM (mg)	HSA (mg)	Trf (mg)	Purity (%)	PF[c]
Initial solution[a]	51.70[d]	9.92	1.08	1.10	31.20	1.77	19.2	1.0
Flowthrough + washing	5.38	5.43	nd[e]	nd	nd	nd	100.9	5.3
Elution	40.40	4.04	1.02	1.00	30.10	1.74	10.0	0.5
Regeneration	0.25	–	–	–	–	–	–	–
Total recovery	46.03	9.47	1.02	1.00	30.10	1.74	–	–

[a] Human plasma diluted 10 times in Mops buffer at pH 7.9 (8.0 mL).
[b] TP: total protein.
[c] Purification factor.
[d] Conductivity of 1759 μS cm^{-1}, pH 7.83.
[e] Values lower than the detectable range of Array Protein System.

tal adsorption data and the fitted curves of the Langmuir and Langmuir–Freundlich models are presented in Fig. 6. The Langmuir model was satisfactorily useful to explain the behavior of HSA adsorption (R^2 value of 0.99). However, this model was not able to fit the adsorption data on human IgG (unrealistic values for adsorption parameters) (Table 5).

For human IgG, the Langmuir–Freundlich isotherm model provided a reasonable fit to the data with an R^2 value of 0.94. The parameter n, a dimensionless exponent related to energy of adsorption, was 2.71 for IgG on the immobilized ω-aminohexyl, indicating that the occupied binding site affects adjacent binding sites positively. The existence of positive cooperation of the binding site for IgG is probably a consequence of the protein–multiple ligand interaction (heterogeneous nature of adsorption).

The apparent equilibrium constants (K_d and K_{dLF}) are a measure of the stability of the complex formed between the HSA and the IgG and the immobilized ω-aminohexyl under specified experimental conditions. The immobilized ω-aminohexyl showed higher affinity for HSA (K_d on the order of 10^{-5} mol L^{-1}) than for human IgG (K_{dLF} on the order of 10^{-3} mol L^{-1}), which are within the range

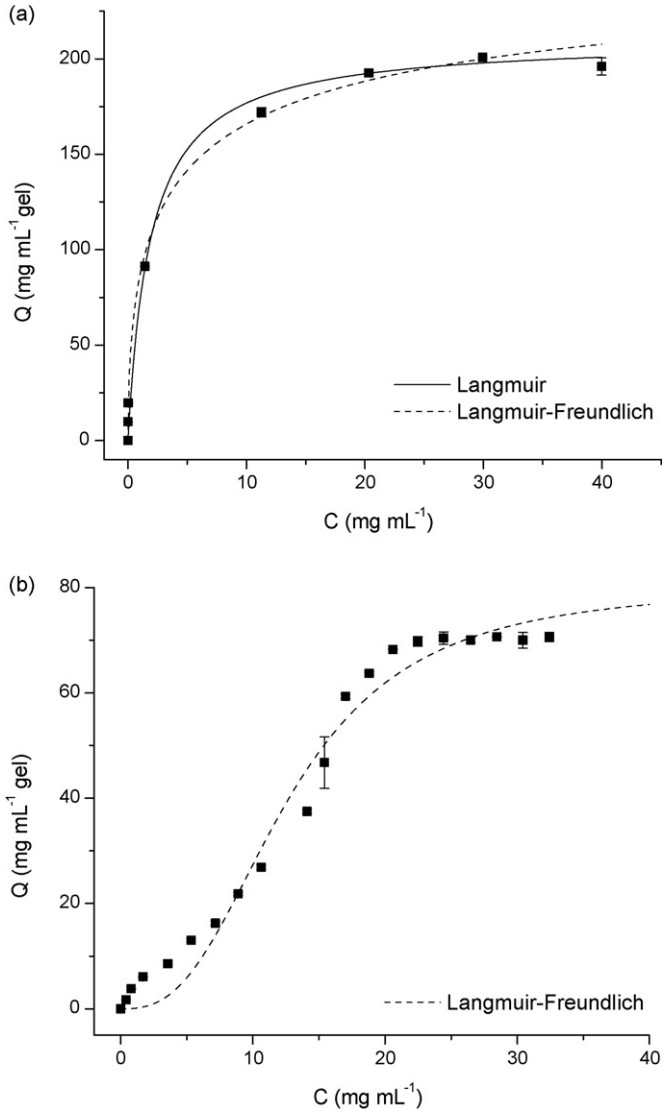

Fig. 6. Experimental adsorption isotherms (symbols) for (a) HSA and (b) IgG and on ω-aminohexyl-bisoxirane-agarose gel with Hepes buffer at pH 6.8 at 25 °C. The solid lines correspond to fitting (nonlinear regression) of experimental data in accordance with the Langmuir and Langmuir–Freundlich models, respectively.

Table 5
Langmuir and Langmuir–Freundlich parameters, correlation coefficient, and variance for the adsorption of HSA and human IgG on ω-aminohexyl-bisoxirane-agarose gel.

Parameters	Isotherm models		
	Langmuir		Langmuir–Freundlich
	IgG	HSA	IgG
Q_m (mg/mL)	–[a]	210.4 ± 6.3	80.3 ± 5.2
K_d (mol/L)	–[a]	(2.9 ± 0.6) × 10^{-5}	–
K_{dLF} (mol/L)	–	–	(6.5 ± 6.4) × 10^{-3}
n	–	–	2.71 ± 0.43
Correlation coefficient	–	0.99	0.98
Variance	–	76.39	19.15

[a] Unrealistic (extremely high) values of adsorption parameters.

of pseudobiospecific ligands. This weaker affinity of immobilized ω-aminohexyl for the human IgG indicates that the interactions between IgG and the free amino group of immobilized aminohexyl are probably nonspecific. The K_d value obtained for HSA (K_d on the order of 10^{-5} mol L^{-1}) is in accordance with the structural analysis developed by Beauchemin et al. [30], which showed that polyamines bind nonspecifically to HSA (H-bonding) via polypeptide polar groups.

The binding affinity shown by human IgG and HSA on ω-aminohexyl-bisoxirane-agarose are weaker than that observed on TREN agarose (dissociation constant on the order of 10^{-6} and 10^{-5} mol L^{-1} for HSA and human IgG, respectively). These differences are probably due to terminal amino groups of the immobilized ligand. TREN is a nonbiological ligand with four nitrogen atoms, three of which are primary in nature and the fourth one is tertiary [31], while ω-aminohexyl is an aliphatic diamine with the length of the alkyl chain of the six carbons. Due to the high amine residue content of TREN, the charge of the free amino groups could probably be very important in the binding of HSA and human IgG, following the order tetramines > diamines. The difference in K_d values obtained for human IgG on TREN and on aminohexyl adsorbents is in accordance with studies developed by Delcros et al., which showed the importance of the charge (number of terminal amino groups) in the binding of polyamines to an antispermine monoclonal antibody [32].

The maximum capacity for HSA obtained for ω-aminohexyl-bisoxirane-agarose (210.4 mg mL^{-1} of gel) was similar to that obtained for TREN agarose (191.7 mg mL^{-1} of gel [12]), but approximately two times higher than the maximum capacity for HSA on histidine-grafted aminohexyl-Sepharose (93.3 mg mL^{-1} of gel; [14]).

The maximum capacity of IgG (80.3 mg mL^{-1} of gel on ω-aminohexyl-bisoxirane-agarose was 2.6 times lower than the value for HSA (210.4 mg mL^{-1} of gel). In fact, the IgG binding capacity is lower because only IgG with pI from 5.5 to 7.5 is adsorbed on this gel. Furthermore, human IgG molecules may cause steric hindrance, decreasing IgG adsorption, and protein-protein interaction (n = 2.71) may form IgG complexes that are not adsorbed onto the immobilized aminohexyl.

4. Conclusion

Negative chromatography was demonstrated to be a successful method to isolate IgG from human serum or plasma. In this work, it was observed that the simple aliphatic amine aminohexyl binds as much serum protein as the complex amine TREN, but the purity of IgG in the former is better (based on IgG, IgA, IgM, HSA, and Trf determined with nephelometric methods and SDS-PAGE analysis).

The IgG recovery in nonretained fractions and the selectivity of the ω-aminohexyl-grafted agarose were influenced by the

nature of the buffer and pH. Under selected conditions, the non-retained IgG was recovered in a single step. No denaturing agents or acidic pH were required to promote nonretention of IgG. The maximum capacity of HSA (principal extracellular protein found at a high concentration in blood plasma or serum) obtained for ω-aminohexyl-bisoxirane-agarose was comparable to that obtained for the TREN agarose gel. Thus, aminohexyl-bisoxirane-agarose constitutes an alternative adsorbent for the isolation of human IgG. The application of ω-aminohexyl-bisoxirane-agarose for purification of human IgG demonstrated that this adsorbent has a great potential for integration into large-scale plasma fractionation processes.

Acknowledgments

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