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High-Performance Liquid Chromatography as a Technique to Measure the Competitive Adsorption of Plasma Proteins onto Latices

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Isotherms of human serum albumin (HSA), human immunoglobulin G (HIgG), and human fibrinogen (HFb) onto a polystyrene (PS)-latex were determined by depletion of protein in the solution, which was either followed by radioactivity measurements or by UV spectroscopy. Different adsorption isotherms for the same protein were obtained when either radioactivity measurements or UV spectroscopy was used as a detection technique. In order to obtain reliable results from competitive protein adsorption experiments, a method based on the use of high-performance liquid chromatography was developed. A strong preferential adsorption of HFb was observed when adsorption studies were carried out with mixtures of HSA, HFb, and HIgG. When adsorption studies were carried out with solutions containing HSA monomer and dimer, a strong preferential adsorption of HSA dimer was also observed.

INTRODUCTION

When solid materials are contacted with blood, proteins usually adsorb at the solid–liquid interface. Several investigators have tried to relate the blood compatibility of materials with the nature and the conformation of the proteins present at the interface (for reviews see Refs. (1–4)). Protein adsorption studies were carried out with single-protein solutions (5–37) and multicomponent systems (22, 38–62) including plasma (45–51, 61, 62), and blood (52–60).

Several methods have been used to perform these studies. These include depletion techniques (5–11, 32–34, 62), infrared spectroscopy (12–15, 22, 61), circular dichroism (8, 16), total internal reflection fluorescence (21, 24), ellipsometry (17, 18), interfacial tension measurements (19, 20, 28, 49, 55–57), electron microscopy (59), and the use of proteins labeled with fluorescent (19, 37, 38) or radioactive (25–36, 39–54, 63) compounds.

From the techniques mentioned above only the use of radiolabeled or fluorescent-labeled proteins and/or depletion techniques seem to be suitable for competitive adsorption studies. Although several investigators claim or assume that the adsorption behavior of labeled proteins does not differ from that of nonlabeled ones, some reports indicate that preferential adsorption of labeled protein may occur (33, 34, 63).

In this study first the depletion of the proteins human serum albumin (HSA), human immunoglobulin G (HIgG), and human fibrinogen (HFb) from single-protein solutions in contact with a PS-latex was followed by radioactivity measurements and UV spectroscopy. Because different adsorption isotherms were obtained, we have studied the feasibility of high-performance liquid chromatography (HPLC) to measure the depletion of single proteins from multicomponent protein solutions after contact with a PS-latex. The competitive adsorption of HSA, HIgG, and HFb as well as the influence of HSA dimer on the adsorption of HSA monomer was investigated using this technique.

MATERIALS AND METHODS

Polystyrene latex was obtained from SERVA (Dow uniform latex particles No.

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41932). The diameter of the particles (0.399 ± 0.006 μm) was verified by scanning electron microscopy. The electrophoretic mobility of the latex particles was measured at 20°C and a pH of 7.35 in a phosphate-buffered saline solution (PBS, 0.01 *M* NaH_2PO_4, 0.01 *N* NaOH, and 0.15 *M* NaCl) with a Rank Brothers Mark II microelectrophoresis apparatus. Based on the results of these measurements a zeta potential of −20 mV was found. The specific surface area *S* ($cm^2\ g^{-1}$) was calculated using the formula $S = 6/\rho d$ in which ρ is the density of polystyrene (1.05 $g\ cm^{-3}$) and *d* the particle diameter in centimeters. The specific area was 1.43 $m^2\ ml^{-1}$ based on a percentage of 10% of solid in the PS-latex.

HPLC. A high-performance liquid chromatograph (Waters Associates) was used with a Toyo Soda Micropak TSK-Gel 3000 SW column obtained from VARIAN. The column contains a porous gel with hydrophilic particles (10 μm diam.) packed in distilled water. The dimensions of the column are 7.5 mm inner diameter and 300 mm in length. A UV spectrophotometer (UV Cord, LKB) equipped with a microflow-through cell was used as a detector.

Proteins: human serum albumin (HSA, crystalline mol wt = 6.6×10^4) was obtained from Sigma (Batch No. A 9511). HSA was first characterized by immunoelectrophoresis (Behring apparatus) which did not show the presence of other proteins. However, when using HPLC the presence of dimer (14%) and some trimer could be shown (see Results).

Human Immunoglobulin G (HIgG, mol wt = 1.69×10^5) was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam. The HIgG was characterized by polyacrylamide gel electrophoresis with a Shannon apparatus and HPLC. With both methods the presence of about 9% (w/w) of a high-molecular-weight component (mol wt = 1.6–3.0 $\times 10^5$) is shown.

Human fibrinogen (HFb, mol wt = 3.4×10^5, grade L 90% clottable) was obtained from KABI, Stockholm.

Purification of proteins. HSA, HIgG, and HFb were further purified by Sepharose 6B column chromatography. The dimer of HSA is almost completely removed. It was not possible to remove the HIgG impurity. A high-molecular-weight compound (mol wt = 10^6) which was present in a small amount in HFb could also be removed from HFb by Sepharose-column chromatography.

Solutions of purified HFb in PBS (pH 7.35, 20°C) were stable for approximately 4 days whereas similar solutions of nonpurified HFb showed precipitates within 1 day. All chromatographic procedures were carried out at 20°C and a pH of 7.35 in phosphate-buffered saline solution (PBS).

Radiolabeling of proteins. Na ^{125}I was purchased from Amersham (IMS 30). The labeling of proteins was carried out with the chloramine-T method (33). After labeling, 75–85% of the radioactivity was incorporated in the protein, which was determined by thin-layer chromatography (TLC) (64). The protein solutions were dialyzed for 16 hr against PBS to remove free label. Experiments were carried out with protein mixtures containing 1–8% of labeled protein. No release of the label could be observed for a period up to 4 days when the proteins were dissolved in PBS at 20°C.

Adsorption of proteins. First the depletion of purified HSA, HIgG, and HFb from single-protein solutions in contact with a PS-latex was followed by either radioactivity measurements or UV spectroscopy. All adsorption experiments were carried out at 20°C in PBS solution with a pH of 7.35.

After the required adsorption time, PS-latex/protein suspensions were centrifuged during 15 min in a Sorvall RC 2-B centrifuge (20,000*g*). The supernatant was isolated and the decrease of the protein concentration was determined by measuring either the radioactivity in the solution with a COBAS type gamma counter or by UV spectroscopy at 280 nm using a Perkin–Elmer 551 apparatus. Extinction coefficients for HSA (0.53), HFb (1.55), and HIgG (1.36) were determined at 280 nm. In those cases where no clear super-

natant was obtained after centrifugation, the supernatant was filtered using a Millipore cellulose acetate filter (pore size 0.2 μm). When clear supernatants were filtered, no detectable change in the protein concentration was observed.

In competitive adsorption experiments the depletion of HSA monomer, HSA dimer, HIgG, and HFb was followed by high-performance liquid chromatography (HPLC) using UV spectroscopy as a detection method. The decrease in solution concentration of each protein was determined by measuring the specific peak areas of the HPLC chromatograms.

RESULTS

An HPLC chromatogram of nonpurified HSA (Fig. 1A) shows three peaks which can be attributed to HSA monomer (3), dimer (3′), and trimer (3″). An amount of about 14% of dimer (peak 3′) is present, and less than 1% of trimer is observed. After purification only a very small amount of HSA dimer was observed (Fig. 1B).

The adsorption of single purified labeled and nonlabeled proteins HSA, HIgG, and HFb from PBS solutions onto PS-latex was measured as a function of time. Within 15 min plateau values were observed which were not changed after 20 hr.

Adsorption isotherms of purified proteins determined by either radioactivity measurements (Rad) or by UV spectroscopy (UV) are given in Figs. 2–4. In all cases isotherms obtained via radioactivity measurements differed significantly from those obtained with UV measurements. Whereas the isotherms (Rad) for all proteins did not reach plateau values in the concentration range studied (0–8 g l^{-1}), the isotherms (UV) reached a plateau at low concentrations of HSA and HFb. For HIgG (UV) a slight increase of adsorbed HIgG is found with increasing HIgG concentration.

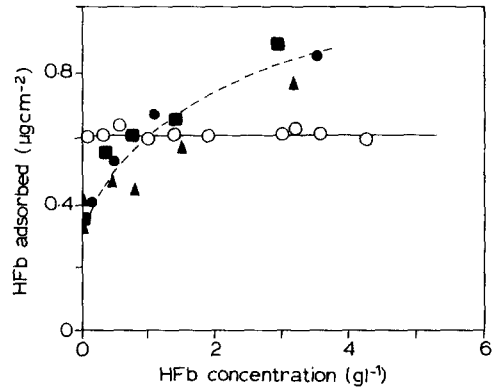

FIG. 2. Adsorption isotherms of HFb (partially labeled) on a PS-latex after 4 hours adsorption time at 20°C, and a pH of 7.35 (PBS) determined by measuring the decrease of protein in the solution using UV spectroscopy (○) and by measuring the decrease of radioactivity in solutions initially containing 8% (▲) 1.6% (●) or 0.8% (■) labeled protein.

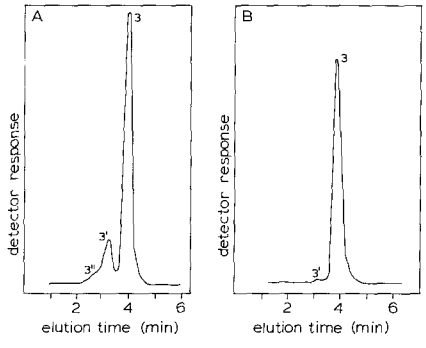

FIG. 1. HPLC chromatogram of unpurified HSA (A), peaks 3 (monomer), 3′ (dimer), and 3″ (trimer), and HSA after Sepharose 6B purification peaks 3 and 3′ (B). Elution buffer PBS, pH 7.35.

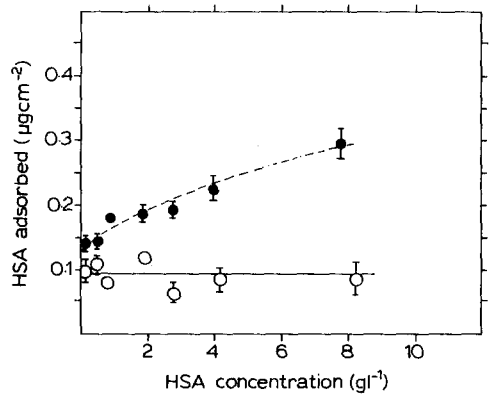

FIG. 3. Adsorption isotherms of HSA (partially labeled 3%) on a PS-latex after 1 hour adsorption time using UV spectroscopy (○) or radioactivity measurements (●).

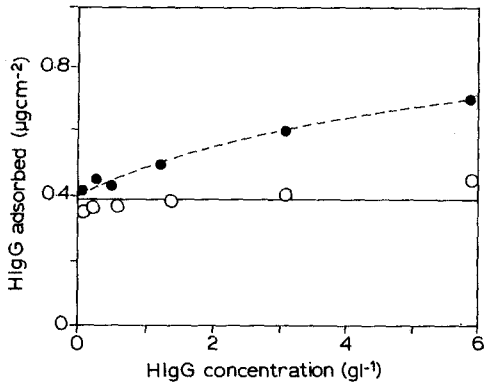

FIG. 4. Adsorption isotherms of HIgG (partially labeled, 3.3%) on a PS-latex after 3 hours adsorption time using UV spectroscopy (O) or radioactivity measurements (●).

When nonpurified HSA is adsorbed onto PS-latex, the percentage of HSA dimer in the total amount of HSA adsorbed, changes with the amount of PS-latex added to a fixed amount of HSA solution (Table I). Only at large latex surface areas (S), the dimer/monomer ratio at the surface and in the solution is equal.

The results of competitive adsorption experiments using combinations of different proteins are shown in Figs. 5–7. The chromatogram of a mixture of HFb (0.37 g l^{-1}) and HIgG (1.8 g l^{-1}) before and after adsorption is given in Fig. 5. Similar data for a mixture of nonpurified HSA (3 g l^{-1}) and HFb (0.3 g l^{-1}) are given in Fig. 6.

In both competitive adsorption experiments HFb shows a strong preferential adsorption. When in these competitive adsorption experiments the concentrations of protein were varied in such a way that after the adsorption HFb is still present in the solution, almost no HIgG or HSA was found on the PS-latex surface. Finally the change in the HPLC chromatograms of a mixture of HSA (2.0 g l^{-1}), HFb (0.45 g l^{-1}) and HIgG (0.76 g l^{-1}) after addition of PS-latex is presented in Fig. 7.

Data obtained from Figs. 5–7 are compiled in Table II. These data were calculated assuming that in all cases HFb was completely adsorbed and that in the competitive adsorption experiments with HFb, HSA, and HIgG no adsorption of HSA occurred.

DISCUSSION

Competitive adsorption. Although several methods were employed to study the adsorption of proteins at solid–liquid interfaces, only

TABLE I

Competitive Adsorption Data of HSA Dimer and Monomer onto PS-latex[a]

		Solution		Surface	
S PS-latex surface area (cm^2)	Perc. of protein adsorbed (%)	HSA final conc. in solution (g l^{-1})	Dimer (%)	Adsorbed amount of HSA ($\mu g\ cm^{-2}$)	Dimer (%) (calc)
447	4.4	0.325	12	0.067	58
447	8.8	0.310	12	0.137	35
894	13.4	0.294	11	0.104	34
894	13.4	0.294	11	0.104	34
1787	29.4	0.240	10	0.114	24
1787	27.5	0.247	10	0.107	24
3575	55.6	0.151	10	0.107	18
3575	54.4	0.155	10	0.105	17
7150	90.0	0.034	13	0.086	14
7150	90.6	0.032	14	0.086	14

Note. Mixtures of 1.4 ml unpurified HSA (14% of dimer) and 0.5 ml PS latex of different concentrations are used. The initial HSA concentration of the mixture is 0.34 g l^{-1}.

[a] Adsorption after 1 hr at 20°C in a PBS solution, pH 7.35.

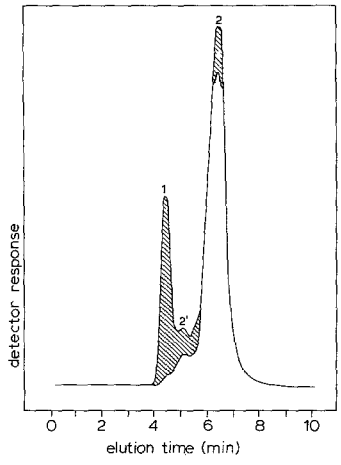

FIG. 5. HPLC chromatogram of a mixture of HFb (0.37 g l^{-1}, peak 1) and HIgG (1.8 g l^{-1}, peaks 2 and 2') in PBS, pH 7.35, 20°C, elution rate 1 ml min^{-1}, elution buffer PBS. Shaded and unshaded area before adsorption, unshaded part after 4 hr adsorption onto PS latex.

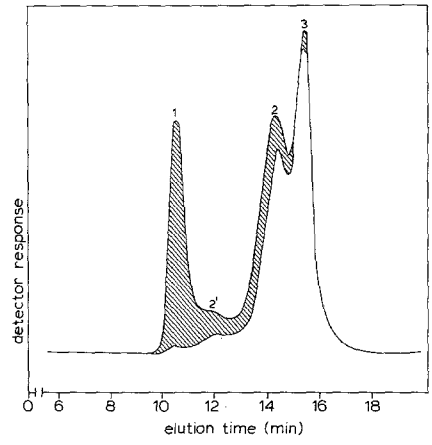

FIG. 7. HPLC chromatogram of a mixture of HFb (0.45 g l^{-1}, peak 1), HIgG (0.76 g l^{-1}, peak 2 and 2') and purified HSA (2.0 g l^{-1}, peak 3) in PBS, pH 7.35, 20°C, elution rate 0.5 ml min^{-1}, elution buffer PBS. Shaded and unshaded area before adsorption, unshaded area after 4 hr adsorption time onto PS latex.

the use of radiolabeling, fluorescent labeling, or depletion techniques seems suitable for competitive adsorption studies.

There is still a controversy whether labeled proteins show the same adsorption behavior as the nonlabeled ones. Van der Scheer *et al.* (34) and Klein Elhorst *et al.* (33) observed a preferential adsorption of iodine-labeled proteins onto a PS-latex. Protein concentrations in the solution after depletion by adsorption onto the latex were determined by radioactivity measurements as well as UV spectroscopy. Grant *et al.* (63) obtained different adsorption values when HSA adsorption onto platinum was studied using ^{125}I-, ^{131}I-, and tritium-labeled HSA. On the other hand the same authors found that the adsorption of HSA on three polymer materials, polyethylene, silicone rubber, and poly(ethylene terephtalate) was independent of the specific activity of the protein in the solution and it was concluded that no preferential adsorption on these polymers occurred. However, the adsorption on the same surface of HSA labeled with ^{125}I differed from that of HSA labeled with ^{131}I when the experiments were carried out under identical conditions.

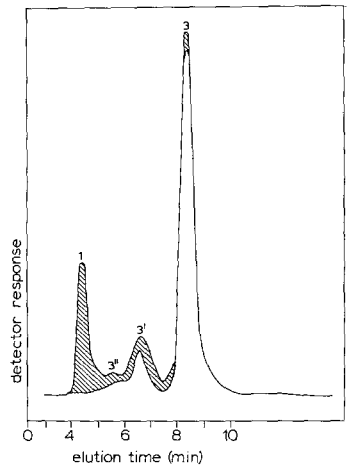

FIG. 6. HPLC chromatogram of a mixture of HFb (0.3 g l^{-1}, peak 1) and nonpurified HSA (3 g l^{-1}, peaks 3, 3' and 3'') in PBS, pH 7.35, 20°C, elution rate 1 ml min^{-1}, elution buffer PBS. Shaded and unshaded area before adsorption, unshaded area after 4 hr adsorption time onto PS latex.

Crandall *et al.* (67) have reviewed the effect of iodination level of proteins on biological clearance behavior, heterogeneity, electrophoretic mobility, metabolic behavior, interchain disulfide bond reactivity, and chromatographic properties. It was concluded that iodination as well as fluorescamine labeling can influence chromatographic and electrophoretic properties.

TABLE II

Competitive Adsorption of Different Proteins onto PS-latex (0.143 m^2)

Buffer volume (ml)	Proteins	Protein conc. in the solution ($g\ l^{-1}$) Before adsorption	Protein conc. in the solution ($g\ l^{-1}$) After adsorption	Protein conc. at the PS latex surface ($\mu g\ cm^{-2}$)
1.5	HFb	0.37	Not detectable	0.4
	HIgG	1.8	1.58	0.2
1.0	HFb	0.3	Not detectable	0.2
	HSA	3.0	2.90	0.07
1.5	HFb	0.45	Not detectable	0.5
	HSA	0.76	0.76	—
	HIgG	2.0	1.80	0.2

Several authors (27–31, 41) have reported that no effect of the presence of a label on the adsorption of proteins onto different surfaces was found. A possible preferential adsorption was determined by carrying out protein adsorption studies with a constant bulk protein concentration and a variation in the percentage of labeled protein.

In order to check whether reliable competitive adsorption data can be obtained when radiolabeled proteins are used in our system, we have compared isotherms calculated from radioactivity measurements for the residual protein in the solution after adsorption with depletion and from measurements using UV spectroscopy. The isotherms of HSA, HIgG, and HFb, respectively (Figs. 2–4) show significant differences dependent on the detection method used. It can be concluded that in such systems the use of radiolabeled proteins leads to unreliable adsorption data.

In order to get reliable competitive adsorption data we have studied the competitive adsorption of proteins onto a PS-latex using depletion studies in combination with HPLC. This technique does not require the modification of proteins. The feasibility of the method is illustrated by the competitive adsorption of HSA, HIgG, and HFb.

When mixtures of either HSA and HFb or HIgG and HFb are contacted with the PS-latex, a strong preferential adsorption of HFb is observed (Table II). Almost no adsorption of HSA or HIgG could be detected, as long as a substantial amount of HFb was present in the solution at equilibrium. It is expected that initially HSA or HIgG are adsorbed onto the polystyrene surface even in the presence of HFb. Since after 1 hr no adsorption of HSA or HIgG is observed, under the condition that HFb is present in the solution, we conclude that initially adsorbed HSA or HIgG will be rapidly exchanged by HFb.

In many adsorption studies nonpurified HSA has been used. Nonpurified HSA contains various amounts of dimer and some trimer (65). It is expected that HSA dimer is preferentially adsorbed as compared to HSA monomer. In preliminary adsorption experiments of albumin onto PS beads, Brooks (66) has obtained evidence for this preferential adsorption. After the addition of small amounts of PS-latex a preferential adsorption of HSA dimer can be clearly observed (Table I). The ratio of adsorbed dimer to monomer decreases with increasing amounts of latex.

Plateau adsorption values. Baszkin and Lyman (49) calculated adsorption values for a side-on or end-on type monolayer adsorption of HSA (0.25–0.90 $\mu g\ cm^{-2}$), HIgG (0.27–1.85 $\mu g\ cm^{-2}$), and HFb (0.18–1.70 $\mu g\ cm^{-2}$). In Figs. 2–4 the amount of adsorbed HSA, HIgG, and HFb on PS-latex (UV) are 0, 1, 0.4, and 0.6 $\mu g\ cm^{-2}$, respectively. The adsorbed amounts of HIgG and HFb are in between the calculated adsorption values for a side-on or end-on type monolayer adsorption. The plateau value of HSA (UV) is lower than expected for a side-on monolayer and consistent with plateau values of HSA adsorption onto PS-latex measured by Norde (5), Van der Scheer (6), and Suzawa (7) under almost the same conditions. Norde assumes a conformational change of HSA during adsorption, rather than the formation of an incomplete layer of unperturbed HSA molecules.

Assuming monolayer adsorption the specific surfaces per molecule for different adsorbed proteins can be calculated from the plateau values (Figs. 2–4). The average specific surface per molecule for HFb, HSA, and HIgG

are 95, 109, and 70 nm^2, respectively. It is striking that although the molecular weight of HFb is substantially higher as compared to HSA the specific surface is even lower. From these results it is concluded that there is more lateral interaction between the HFb molecules present at the surface as compared to the HSA molecules. The lateral interaction between protein molecules may be a factor in the preferential adsorption. A surface charge for the PS particles of less than 2 $\mu C\ cm^{-2}$ has been calculated from zeta potential data (−20 mV). This corresponds with the presence of 1 or 2 negative charges on the latex surface per adsorbed protein molecule in the monolayer. Thus it is expected that in this case the effect of the surface charge on the adsorption values can be neglected.

CONCLUSION

Different adsorption isotherms for the same protein (HSA, HIgG, or HFb) onto a PS-latex are obtained when either radioactivity measurements or UV spectrometry was used as a detection technique to measure the depletion of protein in the solution. Reliable competitive adsorption data of nonmodified proteins can be obtained using the HPLC method. When protein solutions of mixtures of HSA, HFb, and HIgG are contacted with a PS-latex a strong preferential adsorption of HFb is observed. From adsorption experiments with HSA containing both dimer and monomer it is concluded that HSA dimer adsorbs preferentially as compared to HSA monomer.

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