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RAPID HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY ON MICROPELLICULAR SORBENTS

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SUMMARY

Short columns (30 × 4.6 mm I.D.), packed with 2-μm fluid-impervious silica microspheres with surface-bound Protein A or a lectin were used for fast separation and quantitation of immunoglobulins and glycoproteins by biospecific interaction chromatography. With stepwise elution, the total analysis time including column reequilibration did not exceed 3 min. In the assay of IgG with a stepwise change in pH best results were obtained with citrate buffer, which facilitated not only fast but also very sensitive analysis. The calibration curve was linear in the range 0.5–40 μg of human IgG. By using morpholinoethanesulfonic acid–4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–acetic acid buffer with a linear decrease in pH from 6.0 to 4.0 and an increase in magnesium chloride concentration to 200 m*M* for elution, the subclasses of human IgG were separated at 40°C above pH 4.0 in 3 min. Micropellicular concanavalin A and wheat germ agglutinin were used for rapid affinity chromatography of horseradish peroxidase and fetuin, respectively. The results suggest that micropellicular affinity sorbents afford fast and sensitive high-performance liquid chromatographic analysis by biospecific interaction chromatography. Although developed primarily for rapid analysis, the micropellicular Protein A exhibited unexpectedly high adsorption capacity (*e.g.*, 4.5 mg human IgG per ml of wet bed volume). This suggests that such columns could be employed in preparative protein chromatography as well.

INTRODUCTION

Reduction of analysis time with concomitant increase in separation efficiency and detection sensitivity has been a major objective in the development of high-performance liquid chromatographic (HPLC) methods. In the 1960s, columns packed with pellicular sorbents and the use of elevated temperature played an important role in reaching this goal first in the chromatography of small molecules[1–3]. Later, the scope of HPLC was extended to biopolymer separations, and more recently, micropellicular sorbents based on fluid-impervious microspheres of 1.5–7 μm in

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diameter for reversed-phase[4–6] and ion-exchange [7–9] chromatography were introduced for separation of proteins and for rapid peptide mapping.

Protein A from *Staphylococcus aureus*, which binds specifically at the Fc region of immunoglobulin G (IgG), is widely used for purification and analysis of antibodies by biospecific interaction chromatography[10–12]. Affinity chromatography on immobilized Protein A is also used for separation of human IgG subclasses and monoclonal antibodies from mouse ascites and tissue culture supernatants[13–17]. Lectins, in particular concanavalin A (Con A) are used in affinity chromatography for isolation of carbohydrates or glycoconjugates[18,19]. Recently, Con A and wheat germ agglutinin (WGA), immobilized on a microparticulate macroporous support, were used in biospecific HPLC for the separation of a variety of substances having an appropriate carbohydrate moiety[20–24].

The main goal of our present work was to examine the feasibility of using micropellicular stationary phases for rapid biospecific HPLC with Protein A, Con A or WGA covalently bound to the surface of fluid-impervious 2-μm silica microspheres. Stepwise elution was used to isolate and assay IgG and glycoproteins, and gradient elution was used to resolve subclasses of human IgG.

EXPERIMENTAL

Materials

Horseradish peroxidase, fetuin from fetal calf thymus, purified human IgG, α-methyl-D-glucopyranoside, α-methyl-D-mannopyranoside, N-acetyl-glucosamine, tris(hydroxymethyl)aminomethane (Tris), 2-(N-morpholino)ethanesulfonic acid (MES), and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) were obtained from Sigma (St. Louis, MO, U.S.A.). Protein A, concanavalin A, and wheat germ agglutinin were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The tissue culture supernatant containing mouse monoclonal antibody was donated by Prof. M. Constantine-Paton, Department of Biology, Yale University. Octyl sodium sulfate was purchased from Eastman Kodak (Rochester, NY, U.S.A.); the silica microspheres from Glycotech (Hamden, CT, U.S.A.); γ-glycidoxypropyl trimethoxy silane from Dow Corning (Midland, MI, U.S.A.); sodium cyanoborohydride and sodium borohydride from Aldrich (Milwaukee, WI, U.S.A.); and HPLC grade acetonitrile, reagent grade phosphoric acid, magnesium chloride, manganese chloride, calcium chloride sodium chloride and hydrochloric acid from Fisher (Pittsburgh, PA, U.S.A.). Mobile phases were prepared with NanoPure (Barnstead, Boston, MA, U.S.A.) deionized water which was filtered through 0.45-μm membranes and deaerated by sparging with helium before use.

The following instruments were used.

(a) A Model HP 1090, Hewlett-Packard (Palo Alto, CA, U.S.A.) liquid chromatograph with diode-array detector and autosampler. The column effluent passed through the heat exchanger in the diode-array detector before entering the flow-cell. The chromatograms were evaluated by a ChemStation and recorded on a ColorPro graphics plotter. No modification of this instrument was necessary.

(b) A Series 400 pump and Model LC-95 spectrophotometric detector, both from Perkin-Elmer (Norwalk, CT, U.S.A.), were assembled with a heat exchanger coil and a Model 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl loop

detector, injector, and column were kept in a Model DL-8 constant-temperature bath (Haake Buchler, Saddlebrook, NJ, U.S.A.).

(c) Two Model 2150 pumps and a Model 2152 HPLC controller, (all from LKB Instruments, Gaithersburg, MD, U.S.A.), a Rheodyne Model 7125 injection valve and a Kratos (Ramsey, NJ, U.S.A.) Model SF 770 variable-wavelength detector were assembled. The column, heat exchanger coils, a 10-μl Visco Jet micromixer (Lee Co., Westport, CT, U.S.A.) and the injection valve were thermostatted as described for (b) above.

The flow-cells in detectors for (b) and (c) were pressurized and the chromatograms were processed by a Model C-R3A integrator (Shimadzu, Columbia, MD, U.S.A.). Actual pH gradient profiles were determined with the column in place by measuring the pH of 200-μl effluent fractions.

Methods

A suspension of fluid-impervious silica microspheres (d_p *ca.* 2 μm) in an aqueous solution of γ-glycidoxypropyl trimethoxysilane was acidified and heated at 95°C for 2 h[25]. The diol silica thus obtained was oxidized to aldehyde silica by periodic acid at room temperature for 2 h[26]. The proteins (2 mg of Protein A or 5 mg of lectin per g of silica) were attached to the surface by reductive amination using sodium cyanoborohydride at 4°C[26]. The proteinaceous silica microspheres were packed from a slurry in 0.1 *M* phosphate buffer (pH 7.0) into 30 × 4.6 mm I.D. No. 316 stainless-steel columns at 10000 p.s.i.

The 25 m*M* MES–HEPES–acetic acid buffer[27] was prepared by mixing equal volumes of 75 m*M* of each MES, HEPES, and acetic acid. It was titrated to the desired pH with 1 *M* sodium hydroxide. Sample injections were made to coincide with the start of the gradient at the column inlet, as established by tracer experiments described earlier[6].

In experiments where a decreasing pH gradient was used, the starting eluent was 25 m*M* MES–HEPES–acetic acid buffer (pH 6.0). The gradient former was the same buffer, containing 200 m*M* magnesium chloride and titrated to pH 4.0. In the case of stepwise elution, the starting buffer was 100 m*M* sodium citrate (pH 7.4) followed by the same buffer titrated to pH 2.2 with 1.0 *M* hydrochloric acid. After each run, the column was regenerated with the starting buffer at a flow-rate of 1 ml/min in 2 min. The time of equilibration can be reduced by using higher flow-rates. The Protein A column showed no changes upon regular use over a period of three months.

The binding capacity of the Protein A column was determined by frontal chromatography with a solution of human IgG (2 mg/ml) in 100 m*M* citrate buffer (pH 7.4) at a flow-rate of 0.15 ml/min and at 25°C. The experiment was repeated three times and in each case the IgG bound to the column was eluted with 100 m*M* citrate buffer (pH 2.2).

The glycoproteins were bound to the Con A stationary phase from 25 m*M* Tris–HCl (pH 7.0), containing 150 m*M* sodium chloride, 1 m*M* manganese chloride, and 1 m*M* calcium chloride. The stepwise elution was carried out with 50 m*M* α-methyl-D-glucopyranoside in the starting buffer. For the WGA column the starting buffer was 25 m*M* Tris–HCl (pH 7.0), containing 0.15 *M* sodium chloride and 100 m*M* N-acetyl-α-D-glucosamine. The column was reequilibrated in 1 min with the binding buffer at a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

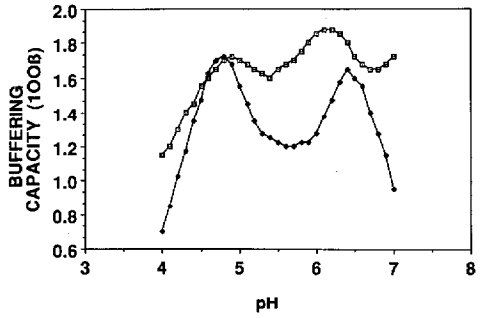
Chromatography on the Protein A column

Purification of IgG on a Protein A column is commonly carried out in two steps: binding under alkaline conditions in the pH range 7.0–9.0, followed by desorption at pH 2.2–3.0 by either stepwise or gradient elution. Whereas stepwise elution is employed for the isolation of IgG in analysis or large scale purification[15–17], gradient elution is mainly used to effect the separation of IgG subclasses[13]. In either case, the enrichment factor of IgG is high, but the acidic eluents used to desorb IgG may cause a loss in immunological activity and reduce the stability of Protein A columns[15–17].

Our goal was to enhance the speed of affinity chromatography of IgG on the Protein A column, to increase the analytical sensitivity, and to elute IgG at a pH as high as possible. According to the literature[13], the subclasses of human IgG are desorbed from Protein A in the pH range 3.0–4.5[13]. For the present work pH 4.0 was selected as the lower pH limit for desorption.

The composition of the buffer system was critical for reaching the goals stated above. The buffer capacities as a function of pH, calculated in terms of β[28] for the MES–HEPES–acetic acid[27] and citrate[13] buffers used in gradient and stepwise elution, are shown in Fig. 1. According to the calculations, the MES–HEPES–acetic acid buffer has a higher and more uniform buffering capacity than citrate in the pH range 4.0–7.0. However, neither buffer in the concentration range 25–50 m*M* was strong enough to elute human or rabbit IgG at pH 4.0 or above.

The interaction between IgG and Protein A is believed to involve all the tyrosine and some of the lysine residues of Protein A and the Fc fragment of IgG[12]. Thus,



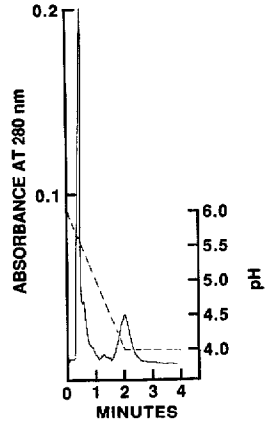
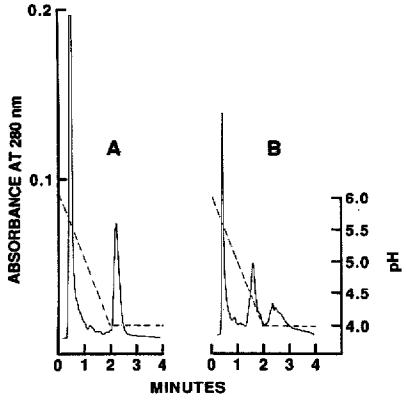


Fig. 1. Calculated buffering capacities as a function of pH: (□) 25 m*M* MES–HEPES–acetic acid and (■) 25 m*M* citrate buffer at 25°C.

Fig. 2. Biospecific interaction chromatography of rabbit IgG on a Protein A column with gradient elution. Column, 30 × 4.6 mm, 2-μm silica based micropellicular Protein A; starting eluent 25 mM MES–HEPES–acetic acid, pH 6.0, gadient former, 25 mM MES–HEPES–acetic acid, 200 mM magnesium chloride (pH 4.0); linear gradient from 0 to 100% B in 2 min; flow-rate, 1 ml/min; temperature, 25°C; sample, 20 μl of serum, diluted 1:40.

besides coulombic interactions, the binding is likely to include hydrophobic interactions as well. For this reason, an increase in buffer concentration and the addition of neutral salts, such as sodium chloride, or ammonium sulfate, to the mobile phase are not expected to facilitate desorption[29]. On the other hand, addition of magnesium chloride to the eluent is expected to reduce electrostatic interactions without promoting hydrophobic interactions between the eluite and the stationary phase. It is believed that magnesium chloride exhibits anomalous behavior and, unlike the other neutral salts, it attenuates hydrophobic interactions between proteins at neutral pH[30,31]. Indeed, a gradient with a magnesium chloride concentration increasing to 200 m*M* and a pH decreasing to 4.0 resulted in the elution of rabbit IgG from Protein A, as shown by the chromatogram in Fig. 2.

In affinity chromatography, extensive band broadening is often attributed to slow kinetics[21,22]. At elevated column temperatures, the kinetics become faster and the efficiency is concomitantly improved[32]. With increasing temperature, the viscosity of the mobile phase decreases, and the faster diffusion rates thus obtained are also expected to improve column performance. The effect of column temperature is illustrated in Fig. 3, which shows chromatograms of rabbit serum at 40 and 60°C. Comparison with the results in Fig. 2 shows that by increasing the temperature to 40°C the IgG peak becomes sharper. At 60°C, another peak appears after that of IgG. It is possible that some IgG undergoes denaturation at this temperature. According to our experiments, a gradient with a pH decreasing from 6.0 to 4.0 and a magnesium chloride concentration increasing from 0 to 200 m*M* at 40°C affords the best results. Under these conditions, the proteins are expected to be more stable than under these commonly used in IgG purification on Protein A. Affinity chromatography of IgG on the micropellicular Protein A column was also carried out with human serum at 40°C according to the same procedure. As seen in Fig. 4, the human IgG subclasses, IgG_1 and IgG_2, were separated in 2 min under the these conditions. As compared with previously published methods[13], our method is not only faster by one order of



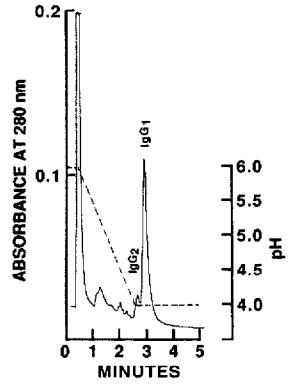


Fig. 3. Biospecific interaction chromatography of rabbit IgG on protein A column with gradient elution at 40°C (A) and 60°C (B). Other conditions as in Fig. 2.

Fig. 4. Biospecific interaction chromatography of human IgG on a Protein A column with gradient elution. Conditions as in Fig. 2, except for 0.5 min isocratic elution, preceding the gradient and at 40°C.

magnitude but also employs milder conditions, *i.e.* a less acidic medium for elution and subsequent recovery of IgG.

Analytical affinity chromatography with stepwise elution is often hampered by extreme changes in refractive index of the mobile phase that occur at the commencement of the desorption step due to the high salt concentration in the debinding buffer. In our case, the baseline drift caused by magnesium chloride interfered with the detection of the IgG peak and greatly reduced the sensitivity of the analysis. This observation prompted us to investigate the requirements of buffer systems of highly sensitive analytical affinity chromatography with stepwise elution.

In stepwise elution, the debinding buffer should have a buffering capacity as high as that of the binding buffer in order to bring about the sudden pH change required to obtain a sharp peak and rapid elution. On the other hand, the binding and debinding buffers should have similar refractive indices to give higher sensitivity of analysis. Buffers such as phosphate–glycine[13], glycine–hydrochloric acid[15], phosphate–acetic acid[16,17], which are frequently employed in affinity chromatography on Protein A columns, fulfil the first requirement but engender excessive changes in refractive index in the desorption step. Therefore, with such buffers large samples size and low detector sensitivity must be used, because the overall sensitivity of the method is quite low. On the other hand, 100 m*M* citrate buffer, which has the buffering capacity of 0.0194 and 0.0186 β at pH 2.2 and 7.4, repectively, is eminently suitable to obtain high analytical sensitivity with stepwise elution of IgG. Furthermore, at low pH, IgG is more stable in citrate[15] than in the other debinding buffers.

The use of citrate buffer in the assay of IgG from human serum and from tissue culture supernatant by high speed affinity chromatography with stepwise elution is illustrated by the chromatograms in Fig. 5. It is seen that IgG appears as a single peak in 2 min after sample introduction. The serum was diluted 40 times and the sample volume was 20 μl in both cases. The response was linear in the range 0.5–40 μg of

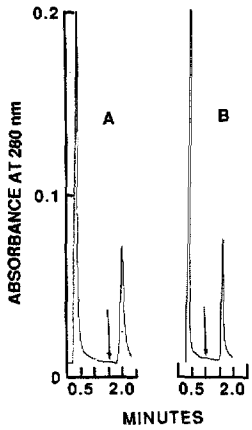


Fig. 5. Biospecific interaction chromatography of human IgG from serum (A) and mouse monoclonal antibody (B) on a Protein A column with stepwise elution. Column, 30 × 4.6 mm I.D., 2-μm silica based micropellicular Protein A; binding buffer 100 m*M* citrate (pH 7.4); debinding buffer, (introduced at the arrow) 100 m*M* citrate (pH 2.2); flow-rate, 1 ml/min; temperature, 25°C, sample 20 μl of tissue culture supernatant or serum, diluted 1:40.

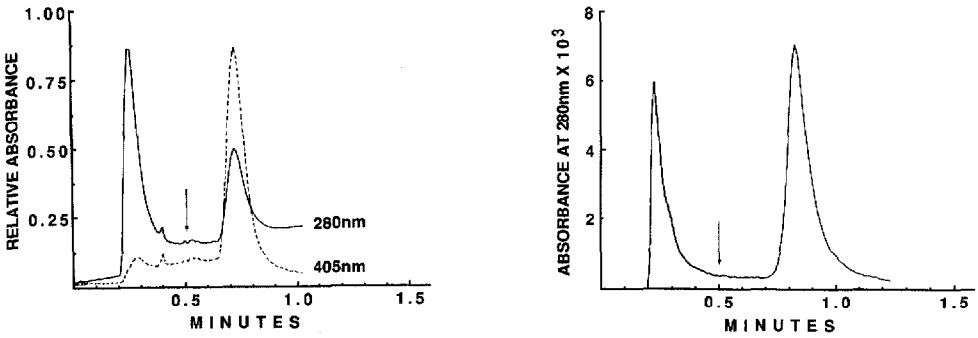


Fig. 6. Biospecific interaction chromatography of horseradish peroxidase on a Con A column by stepwise elution. Column, 30 × 4.6 mm I.D., 2-μm fluid-impervious silica-based micropellicular Con A; binding buffer, 25 m*M* Tris–HCl (pH 7.0)–150 m*M* sodium chloride–1 m*M* manganese chloride–1 m*M* calcium chloride; debinding buffer (introduced at the narrow), 50 m*M* α-methyl-D-glucopyranoside in the binding buffer; flow-rate, 1 ml/min; temperature, 25°C; detector sensitivities, 0.1 and 0.05 a.u.f.s. for 1.0 relative absorbance at 280 and 405 nm, respectively; sample, 20 μg.

Fig. 7. Biospecific interaction chromatography of fetuin from fetal calf serum on WGA column by stepwise elution. Column, 30 × 4.6 mm I.D., 2-μm silica based micropellicular WGA; binding buffer, 25 m*M* Tris–HCl, pH 7.0; debinding buffer (introduced at the arrow), 100 m*M* N-acetyl-α-D-glucosamine in the debinding buffer; flow-rate, 1 ml/min, temperature, 25°C; sample, 20 μg.

human IgG. The correlation coefficient of the calibration curve was 0.995. Therefore, this analytical technique is not only more rapid but also more sensitive than other chromatographic methods[15–17].

The binding capacity of micropellicular sorbents containing Protein A for human IgG was measured by frontal chromatography. It was found that the total human IgG binding capacity of a 30 × 4.6 mm I.D. column was 2.25 ± 0.1 mg, as measured by frontal chromatography at pH 7.4. This represents a capacity of 4.5 mg IgG/ml of bed volume or 3.2 mg IgG/g of dry column packing. The capacity of the column, measured in the presence and absence of 10 mg/ml bovine serum albumin in the IgG solution, was essentially the same, so that non-specific binding did not interfere with the capacity measurement. This IgG binding capacity is about 1/2 or 1/3 of that of commercially available affinity sorbents containing Protein A immobilized on a totally porous silica support[13]. As the binding capacity of the porous and micropellicular sorbents are commensurate for such a large molecule as IgG, the latter type of stationary phase could be useful not only in analytical but also in preparative chromatography.

Chromatography on lectin columns

The potential of silica-supported micropellicular Con A and WGA stationary phases for rapid affinity chromatography was investigated by using commercial horseradish peroxidase and fetuin as sample mixtures, respectively. Since peroxidase contains a protohemin group, which absorbs strongly at 405 nm[8], this protein could be specifically detected at that wavelength. Fig. 6 shows a typical chromatogram of commercial peroxidase on micropellicular Con A silica, while Fig. 7 depicts the chromatogram of fetuin on micropellicular WGA silica. In both cases stepwise elution

was used and the time of the separation was less than 1 min. The total analysis time, including the reequilibration of the column, was about 2 min. Thus, it was much shorter than that reported in literature[20,24]. Moreover, the sensitivity of analysis was also significantly higher.

CONCLUSIONS

The results of this study have demonstrated that micropellicular affinity sorbents are also suitable for rapid biospecific interaction chromatography under appropriate conditions with stepwise elution. IgG could be analyzed with high sensitivity by using a short column, packed with silica supported micropellicular Protein A, within 3 min, including reequilibration time. In a similar fashion, glycoproteins were rapidly analyzed by using micropellicular lectin columns. The binding capacity of a micropellicular Protein A column for IgG was similar to that reported for Protein A, immobilized on totally porous silica. Thus, such columns may be useful, not only for analysis but also for preparative chromatography of proteins and may be expected to facilitate fast separations with high recovery.

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