Journal of Chromatography, 423 (1987) 41–50
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3890

CHARACTERIZATION OF ADSORPTION ON THE STATIONARY PHASE USING HIGH-PERFORMANCE IMMUNOAFFINITY CHROMATOGRAPHY

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(First received May 13th, 1987; revised manuscript received August 6th, 1987)

SUMMARY

Low-level adsorption on the stationary phase has been studied using immunochemical reagents. An immunoaffinity column has been evaluated using affinity-purified radioisotope-labeled monoclonal antibodies. Recovery experiments including continuous immunosorbent monitoring have been performed. Proper characterization of an immunoaffinity separation can result in the recovery of immunologically active material in high yield.

INTRODUCTION

The growing development and production of proteins for both medical and industrial applications presents new challenges for the purification and analysis of these macromolecules [1]. Research on the adsorption of proteins to stationary phases [2,3] represents an important contribution to the improvement of their separation efficiency.

Clinical chemistry today relies heavily on the use of diagnostic kits which contain antibody reagents for analysis [4]. With the aim of greater reproducibility and speed, more and more assays will be automated. One approach to this problem has been to attach these reagents to a stationary phase which can be packed into a high-performance liquid chromatography (HPLC) column. Assays can be performed in a flow injection analysis mode [5], and this process could easily be scaled up to do preparative work. Unfortunately, such immunosorbents have often been associated with poor recovery efficiencies and the isolation of material with low biological activity. This may be due in part to adsorption effects related to

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0378-4347/87/$03.50

unmodified or improperly blocked immunosorbent sites. This general phenomenon will be addressed using immunochemical reagents.

A model for antibody–antigen reactions based on a simple chromatographic method was developed by Sportsman and co-workers [6,7]. They showed that binding constants determined by this method could be extrapolated to binding constants determined in the batch mode (equilibrium). This model has been tested using radioisotope-labeled protein to study low-level adsorption effects. Results from recovery experiments including continuous immunosorbent monitoring will be presented. In this study monoclonal antibodies have been employed because of their more homogeneous properties compared to polyclonal antisera.

EXPERIMENTAL

Materials

Reagents. Mouse monoclonal anti-bovine immunoglobulin G (IgG) was obtained from American Qualex International (La Mirada, CA, U.S.A.). Bovine IgG (bIgG), Cohn fraction V, was purchased from Fluka (Hauppauge, NY, U.S.A.). Human IgG (hIgG) was purchased from Cutter Biological (Berkeley, CA, U.S.A.). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, U.S.A.). Reactigel (6X), a 6% cross-linked agarose bead derivatized with 1,1′-carbonyldiimidazole, was purchased from Pierce (Rockford, IL, U.S.A.). Sephadex G-25 (coarse) and Sepharose 4B were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Radioactive sodium iodide ($Na^{125}I$) was purchased from ICN radiochemicals (Irvine, CA, U.S.A.). Bolton–Hunter reagent for protein iodination [N-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl) propionate] was purchased from Amersham (Arlington Heights, IL, U.S.A.). All other reagents were ACS grade.

Buffer solutions. Non-HPLC solutions were made using distilled, deionized water. HPLC buffer solutions were prepared with doubly distilled water and passed through a 0.45-μm Nylon filter before use. All buffer solutions contained 0.1% sodium azide as a preservative.

Preparative affinity immunosorbent. Approximately 80 mg of bovine IgG were covalently attached [8,9] to 20 g of activated Sepharose 4B. The gel was washed with 300 ml of distilled, deionized water and sucked dry on a 30-ml sintered-glass funnel (coarse). Cyanogen bromide solution (1 ml of 1 g/ml in acetonitrile) was added to a suspension of the gel in 30 ml of 0.33 *M* phosphate buffer (pH 11.9). The mixture was agitated for 2 min on a vortex mixer. The activated gel was then washed with 300 ml of 0.25 *M* phosphate buffer (pH 6) chilled in an ice-water bath, 300 ml of distilled, deionized water and 300 ml of 0.1 *M* sodium bicarbonate–0.5 *M* sodium chloride coupling buffer (pH 8.5). The wet gel was suspended in the protein solution (16 ml of 5 mg/ml) and rotated end-over-end for 46 h at 4°C. Deactivation was carried out by adding 1.5 g glycine to the protein–gel solution and continuing mixing for another 24 h at 4°C. The deactivated gel was washed with 200 ml of water, 200 ml of 0.1 *M* sodium bicarbonate, 200 ml of 0.001 *M* hydrochloric acid, 200 ml of 0.5 *M* sodium chloride and 200 ml of distilled, deionized water. The gel was next washed alternately with 0.05 *M* phosphate

sample buffer (pH 7.4) and elution buffer (pH 2.2) containing 0.15 *M* sodium chloride (phosphate-buffered saline, PBS). Uncoupled protein was determined from the collected washings by absorbance ($A = 1.4$ ml mg^{-1} cm^{-1} at 276 nm). Coupling efficiency was routinely observed to be greater than 90%. The immunosorbent was packed into a 25.0-ml buret which was operated at a flow-rate of 0.3 ml/min at ambient temperature. The preparative column was stored at 4°C in pH 7.4 PBS.

HPLC immunosorbents. Reactigel (6X) was activated according to the manufacturer's instructions, and the excess coupling buffer was removed by decantation. The wet gel (0.75 g) was suspended in 2.0-ml solutions of 0.1 *M* borate buffer (pH 9.5) containing 0.15 *M* sodium chloride (borate-buffered saline, BBS) containing 100 nmol of ligand [bIgG, hIgG, BSA, and blank (no ligand, UN)]. The solution was mixed by end-over-end rotation for 48 h at 4°C. The coupled gel was washed with 50 ml of distilled, deionized water followed by several alternate washes of pH 7.4 and 2.2 PBS. The collected washings were saved for the determination of uncoupled protein. HPLC immunosorbents were packed into columns which were used at 0.5 ml/min. The glass column was operated at room temperature, and the stainless-steel columns were thermostated at 25.4°C. HPLC columns were stored the same way as preparative columns when not in use.

Iodine monochloride. Iodine monochloride was synthesized by generating chlorine gas [10] and passing the gas through a bed of finely ground iodine crystals [11].

Radioisotope-labeled protein. The iodine monochloride method [12] was one method chosen to incorporate ^{125}I into anti-bovine IgG. The protein solution to be labeled was previously dialyzed against three changes of 1.5 l of BBS, pH 9.5, over three days at 4°C. The protein fraction was isolated from the free label by passing the label mixture over a Sephadex G-25 column (21 cm × 1 cm I.D.) equilibrated with PBS, pH 7.4 at a flow-rate of 0.3 ml/min. Fractions of 1 ml were measured for radioactivity. The protein fraction eluted first and was applied directly to a preparative affinity column (described above) also equilibrated with pH 7.4 PBS. Non-anti-bovine IgG proteins and those denatured by the labeling process washed through in the free fraction. Desorption buffer, pH 2.2 PBS, was applied to elute the purified protein. The bound protein fraction was neutralized using an equal volume of 0.3 *M* PBS, pH 7.4, concentrated, and stored at 4°C. The purity of the active fraction was checked by passing a small sample of it through the preparative affinity column (see Fig. 1). The labeled, purified antibody was used to evaluate binding properties (see Figs. 2–4 and Table I).

The Bolton–Hunter Method [13] was also used to label anti-bovine IgG with ^{125}I. The changes from the given procedure were: (i) the protein was dialyzed against pH 9.5 BBS instead of pH 8.5; (ii) the reaction time was 30 min instead of 15 min. The purification was carried out as above. This labeled, purified antibody was used to investigate early affinity column behavior (see Fig. 5).

Fractions of 1 ml were collected in radioisotope experiments and counted using an automatic gamma counter. The bound fractions in these experiments were eluted from columns using pH 2.2 PBS. Following elution of the bound protein, the stainless-steel column was unpacked to determine the activity residing on the

immunosorbent (see Table I). Continuous monitoring of the column activity during experiments was carried out by packing the immunosorbent into a glass column which was placed in the counting well of a manual gamma counter (see Fig. 5).

Apparatus

Much of the apparatus has been described previously [7]. Protein solutions were concentrated with a Biomolecular Dynamics Model MPDC-115 negative-pressure concentrator using a 15 000 molecular mass cut-off dialysis membrane. Equilibrium dialysis was carried out using 12 000–14 000 molecular mass cut-off standard cellulose dialysis tubing purchased from VWR Scientific (Los Angeles, CA, U.S.A.). Preparative affinity columns and size-exclusion columns were monitored with a Dupont Model 835 photometer modified with a larger-volume flow cell (100 μl) and a 280-nm interference filter (10-nm bandpass). High-performance immunoaffinity chromatography (HPIC) experiments were conducted using a stainless-steel (4 cm$\times$0.2 cm I.D.) or glass column (described later), a flow-rate of 0.5 ml/min and a 20-μl sample loop. In order to directly measure the amount of radioisotope-labeled protein residing in the column, a glass column was used. Porous polyethylene plastic discs (Isolab, Akron, OH, U.S.A.) were pushed into the ends of a piece of glass tubing to make a column (4.5 cm$\times$0.25 cm I.D.). The glass column was connected to stainless-steel HPLC tubing using polyvinyl chloride tubing. Radiometric determinations of [^{125}I]IgG were carried out on an LKB Model 80000 automatic gamma counter and a Nuclear Chicago Model 4454 manual gamma counter. The glass column was placed in this latter counter to measure residual radioactivity. Correlation of activity measurements was based on the use of standards comparable in geometry and radioactivity to the samples.

RESULTS AND DISCUSSION

Biospecific affinity chromatography has been used here to prepare immunologically active antibodies in high purity. Fig. 1 shows the affinity chromatogram of previously affinity-purified monoclonal antibody. The free, or inactive, fraction represents less than one half of 1% of the total protein eluted, suggesting that this approach gives high yields of biologically active antibody. This preparative column contains several thousand-fold excess of binding sites compared to the amount of protein applied. The affinity-purified radioisotope-labeled reagent was used to perform the experiments described.

The data in Fig. 2 show the importance of matching the sample size and the column. When 3 pmol of purified antibody (above) were applied to an analytical column (Fig. 2), the observed elution behavior was more complex. The difference in flow-rates alone (0.2 ml/min) was one factor which contributed to the difference in elution behavior (Figs. 1 and 2). The most important factors, however, were the differences in sizes of the columns and the concentrations of binding sites present. The preparative column had a bed volume of 20 ml to which 80 mg of bIgG were covalently attached (4 mg/ml). The analytical column had a bed volume of about 250 μl with 2 mg of immobilized bIgG (8 mg/ml). The ratio of

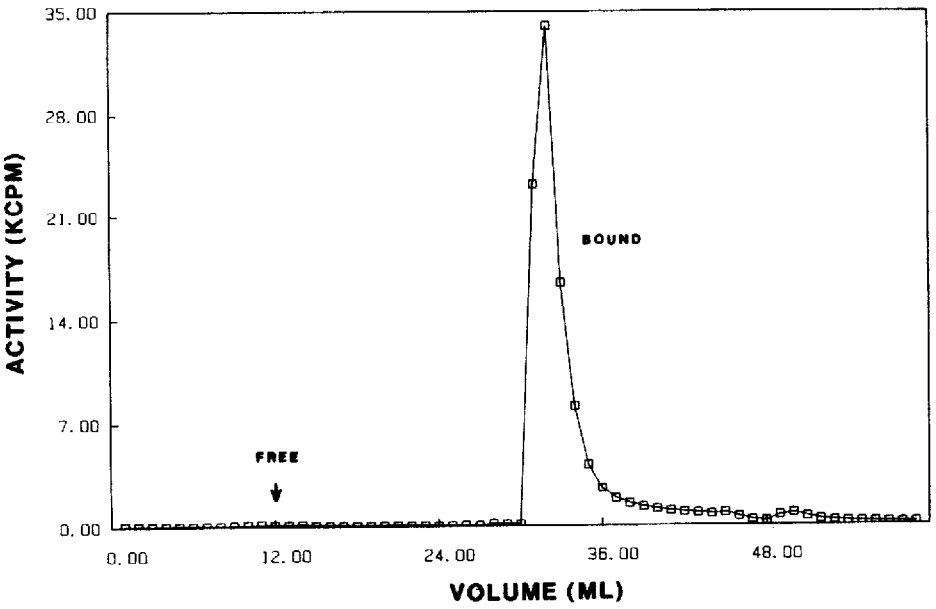

Fig. 1. Evaluation of reagent. Approximately 3 pmol of affinity-purified ^{125}I-labeled anti-bovine IgG (0.2 μCi/mg) was applied to the preparative affinity column (21 cm×1 cm, bIgG–Sepharose 4B, flow-rate 0.3 ml/min).

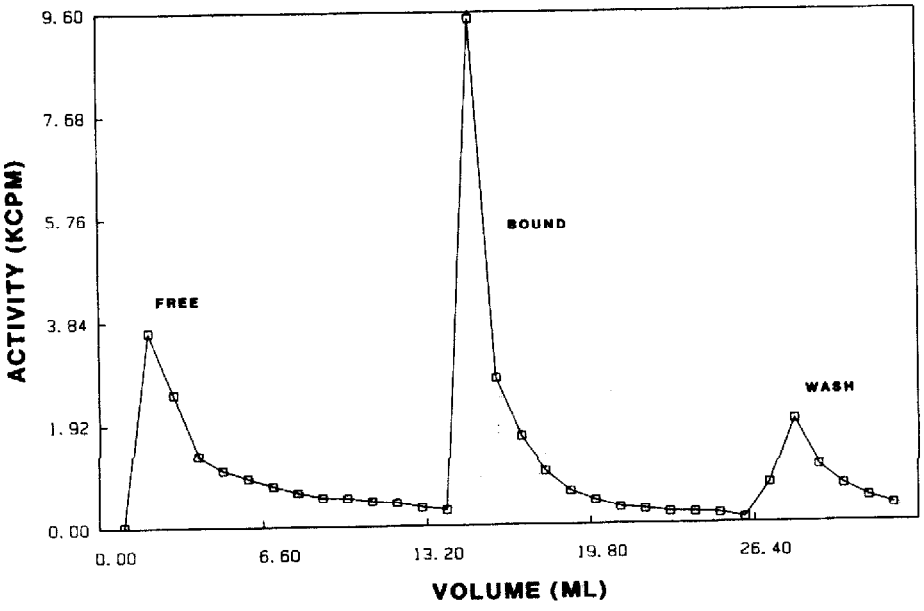

Fig. 2. Analytical-scale chromatography. Approximately 3 pmol of affinity-purified ^{125}I-labeled anti-bovine IgG (0.1 μCi/mg) was applied to an affinity column (4 cm×0.2 cm, undiluted bIgG–Reactigel, flow-rate 0.5 ml/min).

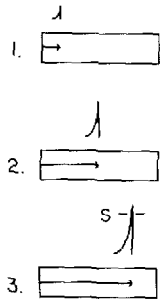

Fig. 3. Elution profile. The following are represented: column (rectangle); progress of wash buffer (arrow); presence of mobile phase antibody dissociated from immunosorbent (peak); solubility limit (S).

binding sites to analyte molecules was much smaller in the analytical column leading to a large "free fraction". The analytical column of Fig. 2 also contained a "wash peak" which appeared as the column was being re-equilibrated with reaction buffer, PBS pH 7.4.

Fig. 3 explains the occurrence of the "wash peak". Upon introduction of antibody molecules into the mobile phase, some molecules formed complexes with stationary phase antigens. For the first line, a step change in the mobile phase to pH 2.2 PBS began to dissociate these complexes producing free mobile phase antibodies. The concentration of antibody molecules increased in a very narrow band at the solvent front of the low-pH buffer as it moved through the column. At some point (line 3) the solubility of the antibody was exceeded, and some molecules adsorbed on the stationary phase. Only after the mobile phase had returned to pH 7.4 PBS did the adsorbed material dissolve and elute. All material recovered from the column, including that from the wash peak, retained essentially full biological activity. The wash peak phenomenon can be eliminated by lowering the concentration of antibody in the mobile phase. Since the wash peak resulted from biologically active material which was bound to the column, the wash peak was combined with the bound fraction for the remaining experiments.

The interaction of a range of antibody concentrations with the analytical column is shown in Fig. 4. The increase in both the free and bound fractions as the amount of antibody introduced into the mobile phase increased agrees with the apparent equilibrium established under the conditions of this experiment. The steady increase in the wash peak corresponds to the elution of greater amounts of antibody from the analytical column where the solubility concentration has been exceeded.

The homogeneity of binding of this monoclonal anti-bovine IgG (also known as IIID12-5) was evaluated previously by Fleenor et al. [14]. However, this was performed by investigating the purity of bIgG which had been affinity-purified using immobilized monoclonal antibody. Density gradient acrylamide gel electrophoresis and isoelectric focusing electrophoresis of affinity purified bIgG, reported by Fleenor et al. [14], showed limited heterogeneity which was considered consistent with isotype homogeneity. Differences in the attachment of the pro-

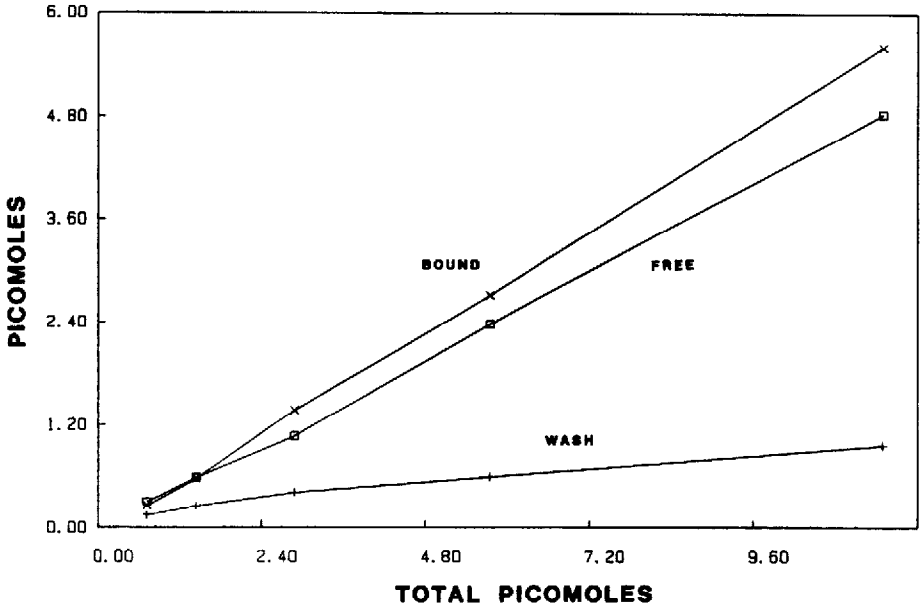

Fig. 4. Antibody partition in various phases. The amount of radioisotope-labeled antibody eluting from the column (conditions described in Fig. 3) in various fractions (free, bound, and wash) versus the amount introduced into the mobile phase is shown.

tein antigen to the immunosorbent support might be expected to contribute to binding heterogeneity.

Optimum performance of immunochemical measurements, especially competitive binding assays (CBA), often necessitates adjusting the number of binding sites in the analytical system. Dilution of the stationary phase with a support material which has minimal interaction with the analyte would be desirable. Table I gives examples of supports which might be used to dilute the specific antigen immunosorbent, bIgG–Reactigel. An initial injection of affinity-purified radiolabeled antibody was introduced via the mobile phase to each immunosorbent.

TABLE I

BINDING PROPERTIES OF IMMUNOSORBENT COLUMNS

One injection of affinity-purified radiolabeled anti-bovine IgG was introduced into the mobile phase of each Reactigel-based immunosorbent (*Materials* section, *HPLC immunosorbents*, column 4 cm × 0.2 cm, flow-rate 0.5 ml/min).

Ligand	^{125}I-labeled anti-bovine IgG (pmol)		
	Free	Bound	Column
bIgG	5.4	98.0	4.3
hIgG	102.8	4.3	0.9
BSA	105.8	2.0	0.2
UN	106.1	1.6	0.2

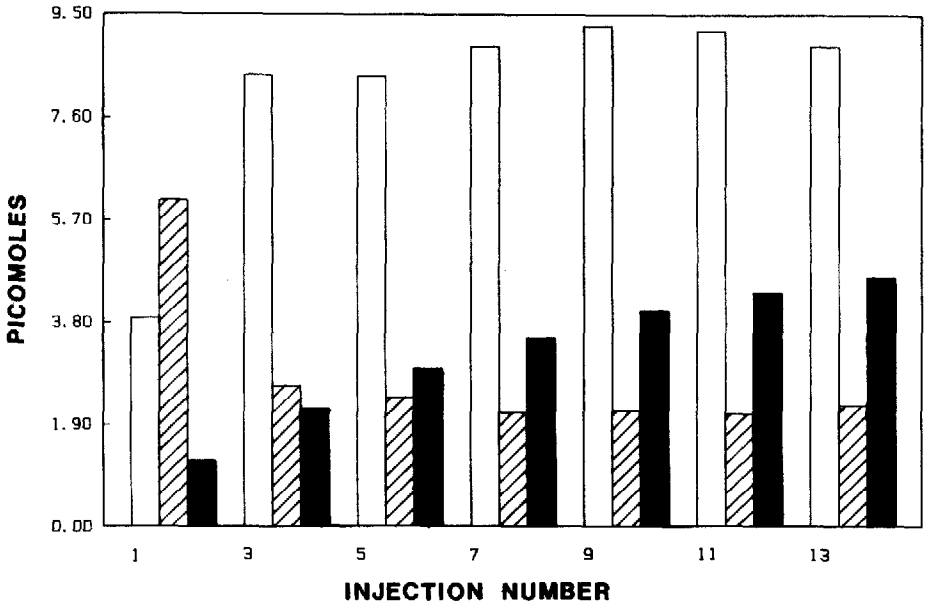

Fig. 5. Affinity chromatography histogram. Approximately 11 pmol of affinity-purified ^{125}I-labeled anti-bovine IgG was repeatedly introduced into the mobile phase (pH 7.4 PBS). The column (4 cm×0.2 cm, undiluted bIgG–Reactigel, flow-rate 0.5 ml/min) was treated with desorption buffer (pH 2.2 PBS) between injections. Column fractions were cumulative. Bar code: (white) free; (hatched) bound; (black) column.

The first exposure of the antibody to this stationary phase resulted in significant (4%) irreversible adsorption. Irreversibly adsorbed material is defined in Table I as that material which remains on the column after repeated washing under normally dissociating conditions. The bIgG–Reactigel (the specific antigen) attracted considerable radioisotope-labeled antibody compared to the hIgG–Reactigel or any of the other supports. This suggests that the irreversible adsorption was not due to non-specific interactions alone. The specific reaction of anti-bovine IgG with bIgG may permit the anti-bovine IgG to approach the stationary phase more closely, thereby enhancing the non-specific adsorption processes. Using the data in Table I, the enhancement ratio of the binding of anti-bovine IgG to bIgG–Reactigel and hIgG–Reactigel, respectively, was estimated as to be 370. By using a radiolabel, it was possible to account for essentially all of the antibody injected. Indeed, less than 2% of the radioisotope-labeled protein was unaccounted for. Within experimental error, all of the modified supports (excluding bIgG–Reactigel) had minimal interaction with the monoclonal antibody. Under these experimental conditions there does not appear to be any advantage to using any one of the stationary phases for dilution purposes in preference to the others.

Fig. 5 shows the effect of repeated injection–elution cycles on the reaction of anti-bovine IgG with the immunosorbent. Although the first five injections (only three are shown for simplicity) gave quite varying ratios of bound to free fractions, the relative amounts of bound and free protein were stable beyond this

stage. The curve traced by the column fractions (irreversibly adsorbed) shows a plateau at about 5 pmol after twenty injections. This quantity appears to be large compared to the bound and free fractions. However, this represents a small fraction (5%) of the total number of sites available in the column. Column saturation experiments have showed that as many as 100 pmol of antibody can bind to this column. The dynamic behavior of affinity chromatography observed here becomes very reproducible beyond several injections. Thus the column can be readily employed in analytical or preparative experiments.

Some of the adsorption behavior observed in the previous experiments might be explained on the basis of a heterogeneous population of antibodies. Both binding and structural heterogeneity could be involved despite the apparent homogeneity of the affinity-purified reagents (Fig. 1).

CONCLUSION

There are a number of factors which contribute to the performance of an immunoaffinity column used for preparative or analytical purposes. The present study has emphasized evaluation of column performance using affinity-purified radioisotope-labeled antibodies. Using this approach it has been possible to demonstrate that essentially all of the material applied to a column can be accounted for. Moreover, after proper column conditioning picomole levels of material can be recovered essentially quantitatively. Even the material found in the wash peak can be recovered and shown to possess high biological activity.

It should be emphasized that the conclusions derived for the particular system studied here will not be uniformly applicable to other immunosorbents or antibody–antigen interactions. However, the present study provides a framework for evaluation of other immunoaffinity systems and demonstrates that reproducible recovery of immunologically active material in high yield is possible.

ACKNOWLEDGEMENTS

We would like to thank the National Institutes of Health, Grant No. DK30718, and American Qualex International for generous financial support.

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