Journal of Chromatography, 266 (1983) 157–162
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 010

DETERMINATION OF IMMUNOGLOBULINS IN BLOOD SERUM BY HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

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SUMMARY

High-performance affinity chromatography columns were prepared by immobilizing protein A from *Staphylococcus aureus* on 10-μm diol-bonded silica. Immunoglobulin-containing samples were injected into the column at pH 7 and eluted by stepwise changes to pH 3. Immunoglobulin G, A, and M standards were tested, but only a small fraction of the latter two was retained on the column. Analysis of 3-μl samples of reference blood sera without pretreatment was performed using immunoglobulin G standards. Good agreement was observed between the immunoglobulin G concentrations measured by this method and by radial immunodiffusion. Chromatography of albumin and electrophoresis of retained serum fractions showed no interference by non-specific adsorption. Chromatographic analysis times were 4 min or less.

INTRODUCTION

High-performance affinity chromatography (HPAC) is potentially useful for the analysis of complex samples, such as biological fluids, with a minimum of sample pretreatment. Relatively non-specific detection methods, such as UV absorbance, can be used if the selectivity of the affinity chromatography column is large enough.

One application of clinical interest is the determination of immunoglobulins in blood serum. There are five known classes of immunoglobulins: immunoglobulins G, A, M, D and E (IgG, IgA, IgM, IgD, IgE). Of these five, only the following three are usually found in significant concentrations in blood: IgG, 7.1–15.3 mg/ml; IgA, 0.6–4.9 mg/ml; and IgM, 0.4–2.1 mg/ml[1]. The concentrations of the immunoglobulins are altered by many diseases[1]. Common methods for measuring immunoglobulins include electrophoresis, radial immunodiffusion (RID), immunoelectrophoresis, nephelometry, and radioimmunoassay[1–3]. HPAC methods for quantitating immunoglobulins should be inexpensive, since the immobilized ligand is reused, as well as simple and rapid.

Immobilized antibodies specific for certain classes or subclasses of the immunoglobulins could be used for HPAC determinations. We have chosen to immobilize a more general ligand, Protein A from *Staphylococcus aureus*, for the determination. Protein A is known to bind to the Fc region of IgG1, IgG2 and IgG4[4,5].

0021-9673/83/$03.00

IgG3, a subclass which normally constitutes 7–9% of the total IgG[6,7], does not react with Protein A[4–6]. The subclasses of IgG have been partially separated on Protein A by affinity chromatography using a pH gradient[6]. It was believed initially that Protein A binds only to IgG[5], but more recent studies have shown that it also binds to at least some subclasses of IgA[4,8], IgM[8,9], and IgE[10]. IgG, IgA, and IgM together constitute 10–20% of the total protein in blood serum[2]. Since Protein A binds to most of these immunoglobulins, immobilized Protein A should be useful for the determination of the immunoglobulin content of blood serum. Bound immunoglobulins can be eluted by a change in pH[5,6,9].

EXPERIMENTAL

Reagents

Protein A and human IgG were obtained from Sigma (St. Louis, MO, U.S.A.). Reference blood sera were obtained from Hyland (Deerfield, IL, U.S.A.). Human IgA (98%) and human polyclonal IgM (98%) came from Calbiochem-Behring (San Diego, CA, U.S.A.). HPLC-grade phosphoric acid (85%) was obtained from Fisher (St. Louis, MO, U.S.A.).

Apparatus

The chromatographic system was described previously[11]. A Bio-Rad 1420B power supply and 155 tube gel cell were used for electrophoresis (Richmond, CA, U.S.A.).

Methods

LiChrospher Si 4000 diol-bonded silica was synthesized and activated with 1,1′-carbonyldiimidazole as described previously[11] with the following modification: 0.050 ml of (3-glycidoxypropyl)trimethoxysilane was used per gram of silica.

Activated silica (0.6 g) was shaken at 4°C with 1.1 mg of Protein A in 2.0 ml of 0.1 *M* sodium phosphate buffer, pH 8.0. After 24 h, 33 μl of ethanolamine were added, and the mixture was shaken for an additional 24 h to remove any remaining active groups. The immobilized Protein A was washed with 2 *M* NaCl and water, then slurry-packed into 5.0 cm × 4.6-mm I.D. columns at 2700 p.s.i. using 0.1 *M* potassium phosphate buffer, pH 7.0.

Chromatography was performed at room temperature. Reference blood sera were injected without pretreatment using 3-μl or 10-μl sample loops. All samples and IgG standards were prepared in the pH 7.0 buffer described below. The weak mobile phase was 0.1 *M* potassium phosphate buffer, pH 7.0, prepared by using reagent-grade K_2HPO_4 and reagent-grade KOH. The strong mobile phase was 0.1 *M* potassium phosphate, pH 3.0, prepared using HPLC-grade H_3PO_4 and KOH. The flow-rate was 1.0 ml/min. Proteins were eluted by stepwise changes to the pH 3.0 buffer.

HPAC peaks were detected at 280 nm, collected by repeated injections, and concentrated by means of an Amicon 12 ultrafiltration chamber and PM 30 membranes (Lexington, MA, U.S.A.). The concentrated fractions were resuspended in pH 7.0 buffer and concentrated a second time; 20–100 μl samples of the concentrated fractions were examined by electrophoresis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was

performed using 7% discontinuous gels at pH 8.9 according to a published procedure[12] which was modified to include 0.1% SDS in solution No. 1 and the electrode buffer solution; 10 cm × 5 mm I.D. gel tubes were used. The gels were stained with Coomassie Brilliant Blue R-250 (Eastman Kodak, Rochester, NY, U.S.A.). Bovine serum albumin, IgG, IgA, and 66,000–264,000 molecular weight standards (all from Sigma) were used as electrophoretic standards.

RESULTS AND DISCUSSION

When Protein A is used for clinical analysis, it clearly is important to know its binding specificity. We examined this by testing the retention of several immunoglobulin classes on our HPAC columns and by electrophoretically separating fractions collected from the columns. Fig. 1a shows that IgA was only partially retained by the HPAC column. This is in agreement with other work which showed that Protein A binds to IgA2 but not to IgA1[8]. Fig. 1b shows that only an insignificant fraction of IgM was retained by the column. This is a surprising result since Protein A columns have been used for the purification of IgM from blood serum[9]. This discrepancy needs to be examined further.

Quantitative results for immunoglobulin analysis could be adversely affected by the non-specific adsorption of other serum compounds if these compounds are eluted by the pH change. Since albumin is the only protein present in blood serum in higher concentration than the immunoglobulins[2], it is most likely to cause such an interference. Fig. 2a shows that no measurable amount of albumin was retained by the HPAC column. Fig. 2b is a chromatogram of a pure IgG standard solution. Very little IgG was unretained. Since Protein A does not bind IgG3, the unretained peak should have been slightly larger, based on the usual fraction of IgG3 present in IgG[6,7]. Perhaps the IgG sample used here had a lower than normal percentage of IgG3.

A five-point calibration curve of peak height (absorbance of retained peak at 280 nm) *vs.* IgG concentration (mg/ml) was prepared using pure human IgG standards. A straight line fitted to the data had slope 0.088 ± 0.003 (90% confidence level) and intercept −0.033 ± 0.032. Reference blood serum samples were analyzed

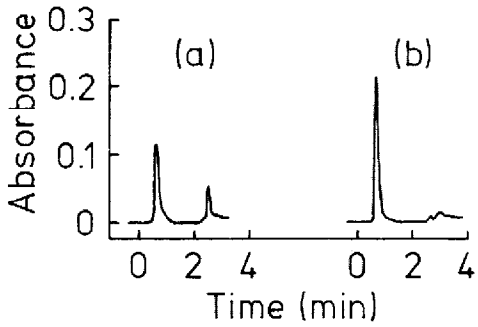

Fig. 1. Analysis of 10-μl samples of 2 mg/ml human IgA (a) and human IgM (b). Approximately 30% of the IgA was retained, while only a trace of IgM was retained. The non-retained fractions were eluted in 0.7 min, while the retained proteins were eluted in *ca.* 3 min by stepwise changes in mobile phase pH. Most of the baseline disturbance at 3 min in the IgM chromatogram was caused by the solvent change.

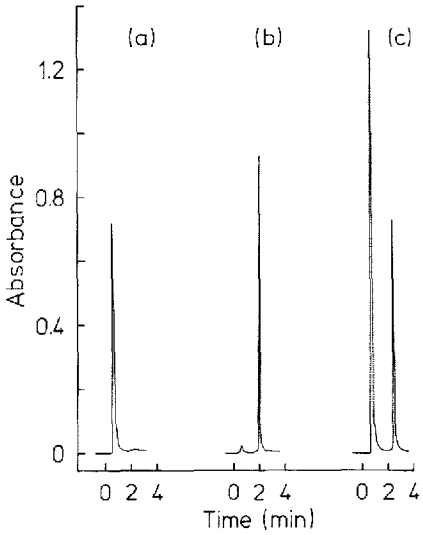

Fig. 2. Analysis of 3-μl samples of 50 mg/ml human albumin (a), 11 mg/ml human IgG (b), and an undiluted reference serum sample containing 8.8 mg/ml IgG (c). The non-retained peak in the IgG chromatogram constitutes *ca.* 3% of the total absorbance of the sample.

without dilution or pretreatment as in Fig. 2c. Table I compares the IgG concentrations for three standard reference sera as determined by the manufacturer by means of RID, with the concentrations determined by HPAC using the calibration curve. There is good agreement between the two sets of values.

The reference sera AI–AIII of Table I were also used as standards to prepare a calibration curve, as before. The fitted line had slope 0.060 $\pm$ 0.006 and intercept 0.040 $\pm$ 0.064. A second set of reference sera, BI–BVI, was quantitated using this curve. The results are shown in Table II. Again, the results of HPAC analysis were in good agreement with the RID values, even for the two samples (BI and BII) which were outside the range of the calibration curve.

Electrophoresis was performed on pooled, concentrated fractions collected from the HPAC column. Using the retained fraction from the pure IgG standards one major electrophoretic band and several very faint bands were observed. The faint

TABLE I

COMPARISON OF REFERENCE SERA ANALYZED BY RID AND BY HPAC WITH IgG AS STANDARDS

Serum sample	*[IgG] (mg/ml)*	
	RID	*HPAC*
AI*	17.50	17.2
AII	8.75	8.9
AIII	2.92	3.6

* This sample only was diluted 1:1 with buffer.

TABLE II

COMPARISON OF REFERENCE SERA ANALYZED BY RID AND BY HPAC WITH OTHER REFERENCE SERA AS STANDARDS

Serum sample	*[IgG] (mg/ml)*	
	RID	*HPAC*
BI	28.15	28.2
BII	22.55	25.7
BIII	16.90	17.0
BIV	11.25	10.8
BV	5.65	6.1
BVI	1.69	1.4

bands may be fragments of IgG[13]. The major band of the non-retained fraction from the IgG standards migrated the same distance as the IgG band in the retained fraction. This band was probably IgG3. A few faint bands were also observed.

The major band from the retained blood serum fractions corresponded to IgG. IgA could not be separated from the IgG, since it has almost the same molecular weight as IgG[1]. A few faint bands were also observed. No band corresponding to IgM was observed.

CONCLUSIONS

The data presented suggest that the results of this HPAC method reflect the IgG concentration of blood serum. However, the specificity of our method needs to be examined further. All serum reference samples contained nearly the same relative concentrations of the major immunoglobulins (74 %, IgG, 15 % IgA, 11 % IgM). The agreement shown in Table II would be expected to be good no matter which immunoglobulins were retained by the column. The good agreement of sera and IgG standards shown in Table I could have resulted if IgG3 was not retained but this difference was made up by the expected partial retention of IgA or IgM.

In spite of these uncertainties, it is clear that this HPAC method may be very useful as a rapid screening method for the immunoglobulins in blood serum. Very small samples were needed —3 μl or less of undiluted serum. Analysis times were of the order of 4 min. The columns had long lifetimes: we have used one column, containing 1.1 mg of Protein A, for more than 200 separations over a period of 4 months.

ACKNOWLEDGEMENTS

This research was supported by the Research Corporation and PHS/NIH Grant No. 2S07 PR07034-15.

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