Journal of Chromatography, 386 (1987) 37–49
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1014

DUAL-COLUMN DETERMINATION OF ALBUMIN AND IMMUNOGLOBULIN G IN SERUM BY HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

DAVID S. HAGE and RODNEY R. WALTERS*,*
Department of Chemistry, Iowa State University, Ames, IA 50011 (U.S.A.)

SUMMARY

High-performance affinity chromatography was used for the simultaneous determination of albumin and immunoglobulin G in serum. Two columns in series, the first containing immobilized anti-albumin antibodies and the other containing protein A for binding immunoglobulin G, were eluted separately at pH 3 by means of a column-switching system. This method gave results in good agreement with commercially available methods, while requiring only 2 μl of serum and 6.0 min per cycle. It was shown that albumin and immunoglobulin G were selectively retained, with little interference from other components, including immunoglobulins A and M.

INTRODUCTION

High-performance affinity chromatography (HPAC) is a highly selective method for the analysis of complex biological samples. By combining the speed of high-performance liquid chromatography (HPLC) with the selectivity of immobilized biological macromolecules, HPAC can be used for analyses of one or a few components in samples, such as blood and urine, with little interference from other components. An area in which this makes HPAC potentially useful is clinical chemistry, where such samples are commonly encountered. One example of the current use of low-performance affinity chromatography in this field is the determination of glycosylated hemoglobins in blood[1]. In this work, we describe the determination of human serum albumin (HSA) and immunoglobulin G (IgG) in serum by HPAC.

HSA is the most commonly determined of the serum proteins[2]. It is the major protein component of serum, making up 50–65% of the total protein content and having typical concentrations of 35–52 g/l (ref. 3). Presently used methods for determining albumin include spectrophotometry, electrophoresis, and single radial immunodiffusion (SRID)[2,3].

The immunoglobulins (or circulating antibodies) consist of five known classes:

* Present address: Drug Metabolism Research, The Upjohn Company, Kalamazoo, MI 49001, U.S.A.

0021-9673/87/$03.50 © 1987 Elsevier Science Publishers B.V.

IgG, IgA, IgM, IgD, and IgE. Of these, IgG is the most abundant in serum, making up 75–80% of the immunoglobulin fraction. Normal levels of IgG vary from 7.0 to 16.8 g/l (ref. 3). IgA and IgM also occur at significant levels in serum with values of 1.4–2.2 and 0.80–1.20 g/l, respectively, but IgD and IgE occur in only trace amounts (*i.e.*, less than 0.2% of the total immunoglobulin fraction)[4]. Immunoglobulin levels are commonly determined by electrophoresis, immunoelectrophoresis, SRID, nephelometry, and radioimmunoassay (RIA)[3–5]. These levels are of interest since they are altered by many diseases and indicate the state of the humoral immune system[3,4].

Possible affinity ligands for these proteins include immobilized anti-HSA antibodies for HSA[6] and protein A for IgG[7]. Protein A, a cell-wall protein of *Staphylococcus aureus*, has been used previously to determine IgG due to its ability to bind to the F_c region of IgG[7,8]. Of the four subclasses of IgG (*i.e.*, IgG_1–IgG_4), protein A binds all but IgG_3, which is not adsorbed or only slightly bound to protein A[8,9]. IgG_3 represents 5–9% of the total IgG[10].

In this paper, these affinity ligands were used in a dual-column system to allow both HSA and IgG to be determined in a single serum sample. Such an approach has the advantage of retaining the high selectivity of each affinity matrix while allowing vastly different components to be determined simultaneously. A general scheme is presented here for the design and optimization of such multi-analyte affinity systems, based on the static and kinetic properties of the matrices and the elution properties of the analytes from the matrices.

THEORY

The kinetic properties of the matrices were determined using the split-peak method described in ref. 11. From ref. 11, the relationship between the flow-rate (F) and the fraction of analyte eluted in the non-retained peak (f) is

$$\frac{-1}{\ln f} = F\left(\frac{1}{k_1 V_e} + \frac{1}{k_3 m_L}\right) \tag{1}$$

where k_1 is the first-order forward mass transfer rate constant for the analyte from the flowing mobile phase to the stagnant mobile phase, k_3 is the second-order rate constant for the adsorption of the analyte onto the matrix, m_L is the total number of binding sites on the column, and V_e is the excluded volume of the column. The above equation assumes irreversible adsorption on the time scale of the experiment (*i.e.*, a small desorption rate constant or large capacity factor, k') and linear elution conditions. Eqn. 1 predicts that a plot of $-1/\ln f$ *vs.* F will yield a straight line with an intercept of zero and a slope equal to $(1/k_1 V_e + 1/k_3 m_L)$.

Since both V_e and m_L are directly proportional to column length and volume, eqn. 1 can be rewritten as

$$\frac{-1}{\ln f} = \frac{C F}{V_{col}} \tag{2}$$

where V_{col} is the empty column volume and C is a constant. Experimentally, C may

be determined using eqn. 2 by injecting pure analyte into a small column of known volume and measuring the fraction eluted in the non-retained peak at various flow-rates.

Note that eqn. 2 is simply the integrated rate equation for a first-order reaction, where V_{col}/F is the reaction time and C is the inverse of the apparent adsorption rate constant. This allows C to be used to determine the time needed to adsorb a given fraction of analyte. This is useful for optimizing sample application conditions.

EXPERIMENTAL

Reagents

Protein A, HSA, human IgG, human IgA, and the affinity-purified goat anti-human IgG, anti-human IgA, and anti-human IgM antibodies were from Sigma (St. Louis, MO, U.S.A.). The human IgM and the rabbit anti-HSA antisera were from Dako (Santa Barbara, CA, U.S.A.). All biochemicals used were of the purest grade available. Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, U.S.A.). The Coomassie Brilliant Blue G-250 was from Kodak (Rochester, NY, U.S.A.), the agarose was from MCB (Norwood, OH, U.S.A.) and the poly(ethylene glycol) (PEG), MW 8000, was from Aldrich (Milwaukee, WI, U.S.A.). The LiChrospher Si-4000 and Si-500 (10-μm particle diameter, 4000-Å and 500-Å pore sizes, respectively) were obtained from Rainin (Woburn, MA, U.S.A.). The Serachem Clinical Chemistry Control sera were from Fisher (St. Louis, MO, U.S.A.).

Apparatus

The chromatographic and data acquisition systems used were the same as described earlier[11], with the addition of a Rainin 7000 switching valve. This valve, placed after the anti-HSA column, contained the protein A column in place of a sample loop. This allowed the protein A column to be switched off-line during part of the analysis. The detector was a Hitachi 100-10 (Tokyo, Japan), operated at 280 nm. The pH of each collected fraction was measured using an Orion 601-A pH/mV meter and a Bioprobe combination electrode (Fisher). A Bio-Rad 1420B power supply (Richmond, CA, U.S.A.) was used for the electrophoresis and a Zeineh soft-laser scanning densitometer (LKB, Gaithersburg, MD, U.S.A.) was used to analyze the gels.

Methods

Diol-bonded LiChrospher Si-4000 and Si-500 were prepared as described previously[12]. The diol contents of the Si-4000 and Si-500 prior to activation were 24 μmol/g and 200 μmol/g, respectively, as determined by the periodate oxidation method[13,14].

The protein A and anti-HSA matrices were prepared using the Schiff base method[15] with the modifications described earlier[11]. Protein A was coupled to the Si-500 by using 10 mg protein A/g LiChrospher Si-500 in the immobilization step, and the anti-HSA was coupled by using 2.0 ml of anti-HSA antiserum/g LiChrospher Si-4000. The protein A had a specific activity of 12 mg human IgG/mg protein A, as determined by SRID[16]. The HSA antiserum had a specific activity of 38.3 μg

HSA/mg protein, as determined by immunoprecipitation[17], and a protein content of 19.6 g/l. The activated silica was placed in 2 ml of pH 5.7 0.10 *M* phosphate buffer/g silica along with 200 mg sodium cyanoborohydride/g silica and was sonicated under vacuum for 15 min. The protein was then added and the mixture shaken at 4°C for 6 days. After 6 days, 500 mg of sodium borohydride/g silica was added to reduce any remaining activated groups[15]. The samples were then centrifuged, washed with 2 *M* sodium chloride and water, and stored at 4°C in 0.1 *M* pH 7.0 phosphate buffer. Part of each sample was washed with water, vacuum-dried at room temperature, and assayed for protein content, using the BCA method[18] with protein A or IgG as the standard, and diol-bonded silica as the blank. The remaining protein A and anti-HSA silicas were then vacuum slurry-packed[19] into minicolumns of a previously published design[20].

The application buffer for both matrices was 0.05 *M* phosphate, 0.05 *M* citrate buffer (pH 7.0). The elution buffer was 0.05 *M* phosphate, 0.05 *M* citrate (pH 3.0). Chromatography was performed at room temperature.

The static adsorption capacity of each matrix was found by continuously applying 0.1 mg/ml human IgG or 0.01 mg/ml HSA to the appropriate matrices, packed in 6.4 mm × 4.1 mm I.D. columns. Both these and all other standard human IgG and HSA solutions were prepared in the pH 7.0 buffer. The breakthrough capacities were determined by integration of the curves[21] and were corrected for the system void volume.

The kinetic adsorption properties of both matrices were studied by the split-peak method previously described[11]. The same conditions were used for the protein A as given earlier[11]. For the anti-HSA matrix, a 12.8 mm × 4.6 mm I.D. column and a 50-μl loop were used over a flow-rate range of 0.5 to 0.75 ml/min.

The elution of human IgG and HSA as a function of pH was studied using a 6.4 mm × 4.1 mm I.D. protein A column and a 12.8 mm × 4.6 mm I.D. anti-HSA column. The columns were first saturated with 0.25 mg/ml human IgG or 0.08 mg/ml HSA, respectively, at 0.50 ml/min. A series of step changes from 0 to 100% pH 3.0 buffer were then made in 5% or 10% increments (*i.e.*, 0.2 or 0.4 pH units) at 15-min intervals. The area of the peak eluted at each pH was determined by planimetry after subtraction of a background run. A fraction was collected at each step change, and the pH was measured.

Quantitation of serum samples was performed at a flow-rate of 1.0 ml/min using anti-HSA and protein A columns, connected in series, and a 10-μl sample loop. The standards were 2.0–14.2 mg/ml HSA and 0.5–4.0 mg/ml human IgG. The serum samples were diluted 1:5 with the pH 7.0 buffer prior to injection. After the non-retained peak had been eluted from both columns, the anti-HSA column was eluted with pH 3.0 buffer, keeping the protein A column switched off-line. After the HSA had been eluted, the protein A column was switched on-line to elute the IgG. Samples of each retained and non-retained fraction from injections of normal serum were collected for later analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% discontinuous vertical slab gels at pH 8.8, using a previously published procedure [22]. Samples of 50–150 μl were applied, using HSA and human IgG as the electrophoretic standards. The gels were stained with Coomassie Blue and analyzed with a scanning densitometer.

TABLE I

PROPERTIES OF PROTEIN A AND ANTI-HSA MATRICES

Matrix	*Protein immobilized (mg/g silica)*	*Coverage (monolayers)**	*Static capacity (mg/g silica)*	*Specific activity (mg/mg)*	*Split-peak constant (C) (s)***
Protein A Si-500	8.6 ± 0.8	0.25	17.7 ± 0.5	2.1 ± 0.2	1.69
Anti-HSA Si-4000	16.5 ± 1.1	1.0	0.90 ± 0.08	(5.5 ± 0.6) · 10^{-2}	<2.8

* Determined using the manufacturer's surface area values of 50 m^2/g for Si-500 and 6 m^2/g for Si-4000, a Stoke's diameter of 100 Å for both protein A[24] and IgG[25], and molecular weights of 42000 and 150000 g/mol for protein A[24] and rabbit IgG[12], respectively.

** The slopes given for the protein A and anti-HSA matrices are for sample sizes of 2% and 20% of the maximum static adsorption capacities, respectively.

SRID was performed as described earlier[23], using 1.5-mm gels and 2-mm sample wells. The gels contained 1% agarose in 0.01 *M* phosphate buffer (pH 7.4), along with 0.85% sodium chloride, 0.02% sodium azide, and 2% PEG. The gels also contained 0.2–1.6 $\mu g/cm^2$ of affinity-purified goat antibodies against either human IgG, IgA, or IgM. The chromatographic fractions applied were preconcentrated 5- to 30-fold on Minicon B-15 clinical sample concentrators (Amicon, Danvers, MA, U.S.A.). After development, the gels were stained with Coomassie Blue.

RESULTS AND DISCUSSION

Static properties of protein A and anti-HSA matrices

The static properties of the protein A and anti-HSA matrices prepared are given in Table I. The protein A Si-500 results were essentially the same as obtained earlier with this immobilization procedure[11]. The immobilization yield for protein A was 86 ± 8% (one standard deviation), and the resulting specific activity was 17% of the initial value claimed by the manufacturer. This rather low specific activity agrees with the findings of earlier work and may be due to such factors as denaturation or improper orientation of protein A on the matrix as a result of the immobilization process[11].

The anti-HSA matrix had an immobilization yield of 43%. This low yield was probably a result of the Si-4000 being saturated with IgG, since approximately monolayer coverage was obtained. This was also suggested by the fact that in using a matrix with a higher surface area, such as Si-500 under the same conditions, virtually 100% of the protein in the antiserum was immobilized.

The specific activity of the anti-HSA matrix, as determined by breakthrough analysis, was 72% of the initial value predicted by immunoprecipitation, assuming a maximum binding capacity of 2 HSA molecules per antibody. This indicated that relatively little anti-HSA activity was lost during the immobilization process.

Elution profiles of human IgG and HSA as a function of pH

The conditions needed for elution of human IgG and HSA from the affinity matrices were determined for a pH elution scheme. The results are shown in Fig. 1.

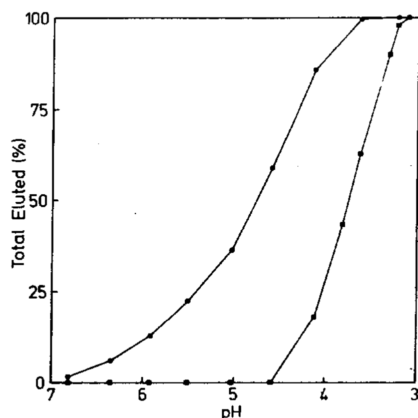

Fig. 1. Total percent human IgG eluted from protein A Si-500 (●) and percent HSA eluted from anti-HSA Si-4000 (■) as a function of pH.

For human IgG, eluted from protein A, all of the IgG was found to be eluted over the range of pH 7.0 to 3.6. This is in agreement with the results obtained previously by others (see ref. 9). Part of the reason for the broadness of this elution range was revealed by taking the derivative of the curve with respect to pH in order to obtain the elution profile as a function of pH. When this was done, two major peaks were found to be present, the largest with a maximum in the elution profile at approximately pH 4.5, and a slightly smaller peak with a maximum at about pH 4.9. These results agree with those obtained by Ohlson for human IgG on protein A Si-4000 (ref. 8) and Duhamel *et al.* for human IgG on protein A Sepharose[26]. In these earlier studies, the peak at the lower pH was identified as IgG_1 and the other as IgG_2[8,26]. Because there were at least two independently eluted species present, the result was that the pH range needed for elution was broadened, as was seen in Fig. 1. Another contributing factor may have been heterogeneity of the immobilized protein A, as discussed previously[11].

A much narrower pH range was needed to elute HSA from the anti-HSA matrix. Fig. 1 shows that all of the HSA was eluted between pH 4.5 and 3.0. The first derivative with respect to pH of the curve showed one peak in the elution profile with a maximum between pH 3.8 and 3.6. This pH range is the same as that of the N-F transition of serum albumin, during which the protein undergoes a reversible conformational change[27], suggesting that this might be the mechanism by which the HSA–anti-HSA complex dissociates.

In comparing the human IgG and HSA curves in Fig. 1, significant overlap of the two was found to occur, particularly in the pH range of 4.5 to 3.5. The maximum difference in the two curves was 68% at pH 4.1, where 18% of the HSA and 86% of the human IgG were eluted. Since the number of moles of HSA in serum is typically nine times larger than that of IgG, this difference was not sufficient to resolve these two components quantitatively on an anti-HSA–protein A dual-column system by a series of pH step changes or a pH gradient alone.

Selection of operating conditions

Because a pH step change or pH gradient elution scheme was found to be insufficient to resolve IgG and HSA totally on an anti-HSA–protein A system, other means had to be found. This was accomplished by using a column-switching system in which the protein A column replaced the loop on an injection valve and followed the anti-HSA column. In a typical analysis, the sample was injected into the pH 7.0 application buffer, with both columns on-line, to allow the sample to pass through both columns and the HSA and IgG to be adsorbed. Once the non-retained peak was eluted, the protein A column was switched off-line, and pH 3.0 buffer was applied to elute the HSA. The protein A column was later switched on-line to elute the IgG. Finally, pH 7.0 buffer was again applied and the cycle repeated. Using this system, it was possible to adjust the resolution between the IgG and HSA peaks to any desired value by changing the time at which the protein A column was brought back on-line.

An advantage of this system was that, by having the sample pass through the anti-HSA column first, it was possible to reduce interference in the IgG determination due to HSA adsorption on protein A. This was of concern, since in previous studies protein A was shown to bind to porcine, canine, and feline albumin[28]. To test for adsorption by HSA on protein A, injections of normal serum were made into a 10 cm × 4.1 mm I.D. protein A Si-500 column, and the retained fractions were collected. SDS-PAGE was then performed on these fractions. Three bands were seen in the retained sample. The two major bands were found to correspond to the H and L chains of an IgG standard and the third band matched that produced by an HSA standard. Integration of the area under each band with a scanning densitometer revealed that up to 9% of the total area was in the HSA band of the sample[29]. This indicated that a significant amount of HSA had been adsorbed on the protein A column. By placing the anti-HSA column first in the dual-column system, an attempt was made to minimize this effect by removing any HSA present before the sample had passed through the protein A column.

Once the elution scheme and column order had been decided, it was necessary to determine the column sizes needed to retain IgG and HSA quantitatively under normal operating conditions. Two parameters were considered: the column capacity and the adsorption kinetics. The kinetics were particularly of interest, since they have been shown to be limiting factors in adsorption for both protein A[11] and immunoaffinity HPAC matrices[30–33]. This parameter was determined using the split-peak method described earlier. The values of the split-peak constant (C) obtained are given in Table I.

In determining the required column sizes, a flow-rate of 1.0 ml/min and a sample size of 30 μg IgG and 100 μg HSA (*i.e.*, a 10-μl injection of a 1:5 dilution of normal serum) were assumed to be typical operating conditions. The column volume needed to give a column capacity equal to the sample load was determined from the static capacity values in Table I. The minimum value of V_{col} required by the adsorption kinetics was calculated from eqn. 2, using the values of C in Table I, the given flow-rate, and assuming 99% retention (*i.e.*, f = 0.01).

The results are sumarized in Table II. For the protein A matrix, the minimum value of V_{col} required to give a sufficient sample capacity was only 4.5 μl. However, the minimum value of V_{col} needed to kinetically adsorb the IgG was about 30 times

TABLE II

COLUMN SIZE REQUIREMENTS FOR ANTI-HSA AND PROTEIN A MATRICES

	Minimum column volume (μl)	
	*Static**	*Dynamic***
Protein A Si-500	4.5	130
Anti-HSA Si-4000	230	<220

* Based on a sample made up of 30 μg IgG and 100 μg HSA. The results were calculated from the static capacity data in Table I using packing densities of 0.38 g/cm^3 for Si-500 and 0.49 g/cm^3 for Si-4000.

** Based on a flow-rate of 1.0 ml/min, the split-peak kinetic data in Table I, and a minimum retention of 99%.

this value, or 130 μl, making adsorption kinetics the limiting factor in determining column size for this matrix. For the anti-HSA matrix, both the column capacity and adsorption kinetics terms were approximately the same, making both important factors in determining column size. From these two factors, a minimum column volume of 230 μl was calculated for the anti-HSA matrix. In order to allow for larger samples than assumed here, such as might occur when abnormal serum is tested, column volumes of 160 μl for the protein A and of 530 μl for the anti-HSA were actually used (*i.e.*, column sizes of 12.4 mm × 4.1 mm I.D. and 40.4 mm × 4.1 mm I.D., respectively).

This system was tested, and the cycle time was optimized, by injecting standards containing 80 μg HSA and/or 20 μg human IgG. Some typical chromatograms are shown in Fig. 2. Injection of HSA (a) gave a single retained peak which was

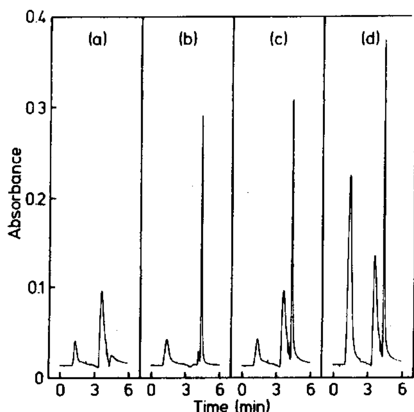

Fig. 2. Chromatograms obtained after injections of (a) HSA, (b) IgG, (c) HSA plus IgG and (d) normal serum into the dual-column system. The event sequence used was: 0.00 min, switch from pH 3 to 7 buffer; 0.50 min, sample injection; 2.25 min, protein A column switched off-line, switch to pH 3 buffer; 4.00 min, protein A column switched on-line. The samples and chromatographic conditions used were the same as described in the text. The small peak at 1.4 min in (a–c) was due to the solvent change from pH 7 to 3 plus IgG_3 in (b–c).

eluted from the anti-HSA column, and injection of IgG (b) gave a single retained peak, eluted from the protein A column. A sample containing HSA and IgG (c) gave a chromatogram that was essentially the same as the sum of those obtained with the HSA and IgG standards. By using the elution times and peak-widths obtained with these standards, as well as the void times for the system and the time required to switch from one buffer to another, a total cycle time of 6.0 min for the separation was obtained, using the event sequence given in Fig. 2. Under these conditions, the resolution (R_s) between the HSA and IgG peaks was 1.0. By delaying the time at which the protein A column was brought back on-line, it was possible to obtain baseline resolution ($R_s \geqslant 1.5$) in a cycle time of less than 7.0 min.

Using this elution scheme, the HSA peaks obtained were typically broader than those for IgG. An attempt was made to reduce the HSA peak-width by using more acidic elution conditions. By going to pH 2.0, it was possible to decrease the HSA peak width by 50% and the IgG peak width by 20%. However, at this pH, the total cycle time actually increased, since at pH $\leqslant$ 2.6 the time required to regenerate the protein A column with pH 7.0 buffer became longer than the value of 30 s required at an elution pH of 3.0. Because of this effect, the elution pH was kept at 3.0 in order to minimize the cycle time while allowing both HSA and IgG to be quantitatively eluted.

Quantitation of HSA and IgG in serum

Injections of a series of protein standards, containing 20–140 μg HSA or 5–40 μg of human IgG, were used to calibrate the system. These standards covered the expected range of 70–104 μg HSA and of 14–34 μg IgG for a 10-μl sample of a 1:5 dilution of normal serum. The event sequence used was chosen to give a value for $R_s \geqslant 1.5$ between the HSA and IgG peaks so that both peak heights and areas could be determined. The responses were linear up to 100 μg for IgG and 140 μg for HSA. Correlation coefficients of 0.996 to 0.997 over 7–9 points were obtained for all of the calibration curves. From these curves, the limit of detection for IgG at a signal-to-noise ratio (S/N) of 3 was estimated to be 0.68 μg when either peak-height or peak-area measurements were used. For a 1:5 dilution of a serum sample, this is equivalent

TABLE III

RESULTS FOR CONTROL SAMPLES IN A PROTEIN A–ANTI-HSA SYSTEM

Sample	*Conc. IgG (g/l) ± 1 S.D.*			*Conc. HSA (g/l) ± 1 S.D.*		
	Peak height results	*Peak area results*	*Ref. values**	*Peak height results*	*Peak area results*	*Ref. values***
Normal serum	9.5 ± 0.6	9.7 ± 0.2	8.6 ± 0.3 to 9.3 ± 0.4	43 ± 1	47 ± 1	40 ± 3 to 44 ± 4
Abnormal serum	5.6 ± 0.2	5.8 ± 0.5	5.4 ± 0.3 to 5.7 ± 0.4	26 ± 2	26 ± 2	21 ±3 to 27 ± 2

* The reference values given are the low and high assay values provided by the manufacturer.

to an initial serum concentration of 0.34 g/l. The limit of detection at a S/N of 3 for HSA was 2.6 μg, using peak-heights, and 3.5 μg, using peak-areas, corresponding to initial serum concentrations of 1.3–1.8 g/l, respectively.

A series of injections of control sera, diluted 1:5 with pH 7.0 buffer, was made. A sample chromatogram is shown in Fig. 2d. The results are shown in Table III for serum samples containing normal and abnormal levels of IgG and HSA. The data given are the average of 7–8 serum injections for IgG and 3–5 injections for HSA. No significant difference was noted for either IgG or HSA when comparing the peak-area and peak-height results. As has been noted previously, the results obtained for IgG with this method agreed well with those of commercial SRID[7] and nephelometric assays. All experimental values were within one standard deviation of the range of control values given by the manufacturer. Similar agreement was found between the experimental values for HSA and the control values from commercially available spectrophotometric and colorimetric dye-binding assays. The precision of the HSA and IgG experimental results was also comparable to that of the control methods. For instance, the HSA peak-height and peak-area results gave average precisions of 4.7% and 4.6% (1 R.S.D.) for the two samples tested, while the commercial methods had a range of 1.8% to 6.3%. The IgG peak-height and peak-area results had average precisions of 4.5% and 5.1%, compared to values of 3.7–11.9% obtained with the other methods.

The reproducibility of the results obtained with this system was examined using a series of 45 injections of normal control serum. The heights and areas of the HSA peaks were found to vary by ±4.0% and ±3.4%, respectively, while the heights and areas of the IgG peaks varied by ±2.6% and ±3.6%, respectively.

A similar set of injections was used to determine the lifetime of the system. No signs of column deterioration (*e.g.*, abnormally-shaped peaks, increasing/decreasing peak-heights or peak-areas) were observed in over 120 injections into the anti-HSA column. These injections included 60 standard samples and 60 serum samples. This is a much longer lifetime than the typical 20–25 column cycles, reported for similar high-performance immunoaffinity matrices under acidic elution conditions[34]. This is probably a result of the fact that only a mildly acidic elution buffer of pH 3.0 was used here instead of a pH of 2.0 to 1.0, as commonly used with such matrices[34]. The protein A column showed no signs of deterioration after more than 170 injections, including 60 standards and 110 serum samples. This indicates that the immobilized protein A was very stable, as has been noted previously[7,11].

The protein A and anti-HSA matrices, prior to packing, were also found to be quite stable. For example, when stored in pH 7.0 buffer at 4°C, the protein A matrix showed no change in its characteristics over a period of at least 18 months and the anti-HSA matrix over a period of at least 6 months.

The purities of the IgG and HSA peaks were examined by SDS-PAGE. Using injections of normal serum, only one band was observed in the fraction eluted from the anti-HSA column. This was identical to that given by an HSA standard. The fraction eluted from the protein A column contained two bands. These matched the bands for the H and L chains of a human IgG standard. Analysis of the gels by scanning densitometry showed no detectable amount (*i.e.*, < 0.5% of the total area) of IgG in the HSA peak or HSA in the IgG peak. Also, no other bands in either fraction were detected with more than 99% of the integrated densitometer scans

being due to HSA in the HSA peak and the H and L bands in the IgG peak.

SDS-PAGE of the non-retained normal serum fraction showed that no detectable amount of HSA (*i.e.*, $<0.5\%$ of the total HSA collected) was present. The relative amount of IgG in the non-retained peak was determined to be 8% by SRID. This agrees with the typical IgG_3 levels in normal serum of 5–9%[10].

The levels of IgM and IgA in the IgG and non-retained peaks were also determined by SRID. These were of interest since IgM and IgA have the ability to bind protein A[9], making them possible interferences in the determination of IgG. From the SRID results, more than 95% of both the IgM and IgA was found to be eluted in the non-retained peak. These levels agree with those obtained previously on a similar protein A HPAC system[8] but are much greater than would be predicted, based on the IgM and IgA levels of 66% and 30%, respectively, that are capable of binding to protein A[9]. A possible explanation for this difference is that IgA and IgM may have exhibited a split-peak effect on the protein A matrix, similar to that seen for IgG[11], but with slower adsorption kinetics. This slower adsorption could be the result of the larger size[4] and slower diffusional properties of IgM and IgA compared to IgG, or the fact that IgM and IgA may have a different mechanism of binding to protein A than IgG (see ref. 9).

CONCLUSION

The data presented show that this system was effective in selectively quantitating IgG and HSA in serum with results for both normal and abnormal samples comparable to those obtained with commercial methods. Some potential advantages of this method over others include its speed of analysis, with the determination of both IgG and HSA being possible in 6.0 min, in comparison to hours or days for electrophoretic and immunodiffusion techniques, and the small sample requirement of only 2 μl of serum per injection. The selectivity of the system also makes it subject to fewer interferences than are encountered with the usual electrophoretic and spectrophotometric methods[35]. Moreover, this system may be easily automated. This can be done by using a system such as that described in ref. 34, with the addition of an automatic valve for column selection. All of these characteristics suggest that this method should be useful for the rapid, routine analysis or screening of serum samples.

Another advantage of this method is that the ratio of HSA–IgG in a sample can be obtained without prior calibration of the system. This can be calculated by using the absorptivities of IgG and HSA at the detection wavelength along with their peak areas and is analogous to the determination of the albumin–globulin ratio[4]. Such ratios have the advantage of being more sensitive than absolute measurements in detecting diseases where the level of one component increases and the other decreases. For example, the ratio of HSA–IgG could be especially useful in the diagnosis of liver disease and chronic infections, where HSA levels are typically decreased and IgG levels increased[4].

One possible limitation of this technique is that, since IgG_3 appears not to be retained, differences in the relative IgG_3 content of the samples and standards can give rise to slight errors in the IgG levels found. This can be minimized by using IgG standards prepared from pooled human serum, such as has been recommended for use with other immunoglobulin assays[36], in order to obtain standards with average

IgG_3 levels. Alternatively, anti-human IgG antibodies could be used in place of protein A as the affinity ligand.

The effect of several potential interferences for this method were examined. Two of these were IgM and IgA, possible contaminants in the IgG determination, due to their ability to bind protein A. However, they did not interfere in this method since more than 95% of both components were eluted in the non-retained peak, possibly as a result of slow adsorption kinetics. Another interference studied was HSA, also a contaminant in the IgG determination, due to its adsorption on protein A columns. This interference was removed by placing the anti-HSA column first in the system, a method which was shown to remove all of the HSA before it reached the protein A column. This demonstrates the potential usefulness of affinity columns in removing undesirable sample components, a technique referred to as "negative" affinity chromatography[37], in analytical applications of HPAC or HPLC in general.

As previously discussed, this dual-column system was not only effective in removing HSA as a contaminant, but was also useful in determining sample levels of both HSA and IgG. The use of such multicolumn or multidimensional systems has several potential advantages for chromatography in general[38]. For HPAC this technique is particularly promising, since one of the disadvantages of HPAC is that it can normally be used to detect only one or a few similar components at a time. The multicolumn approach given here is one way in which this limitation can be overcome by allowing several different analytes to be determined with the same system.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation under Grant CHE-8305057. D.S.H. was supported by an American Chemical Society Analytical Division Fellowship from the Proctor and Gamble Company.

REFERENCES

1 A. K. Mallia, G. T. Hermanson, R. I. Krohn, E. K. Fujimoto and P. K. Smith, *Anal. Lett.*, 14 (1981) 649.
2 T. Peters, Jr., G. T. Biamonte and B. T. Doumas, *Sel. Methods Clin. Chem.*, 9 (1982) 317.
3 A. Kaplan and L. L. Szabo, *Clinical Chemistry*, Lea and Febiger, Philadelphia, 1979, Ch. 5.
4 K. E. Blick and S. M. Liles, *Principles of Clinical Chemistry*, Wiley, New York, 1985, Ch. 6.
5 K. R. Cochrum, in H. A. Harper, V. W. Rodwell and P. A. Mayes (Editors), *Review of Physiological Chemistry*, Lange, Los Altos, CA, 1977, pp. 596–608.
6 S. Ohlson, L. Hansson, P.-O. Larsson and K. Mosbach, *FEBS Lett.*, 93 (1978) 5.
7 S. C. Crowley and R. R. Walters, *J. Chromatogr.*, 266 (1983) 157.
8 S. Ohlson, in I. M. Chaiken, M. Wilchek and I. Parikh (Editors), *Affinity Chromatography and Biological Recognition*, Academic Press, New York, 1983, pp. 255–256.
9 R. Lindmark, K. Thoren-Tolling and J. Sjöquist, *J. Immunol. Methods*, 62 (1983) 1.
10 C. Papadea, I. J. Check and C. B. Reimer, *Clin. Chem.*, 31 (1985) 1940.
11 D. S. Hage, R. R. Walters and H. W. Hethcote, *Anal. Chem.*, 58 (1986) 274.
12 R. R. Walters, *J. Chromatogr.*, 249 (1982) 19.
13 S. Siggia and J. G. Hanna, *Quantitative Organic Analysis*, Wiley, New York, 1979, pp. 42–43.
14 A. A. Woolf, *Anal. Chem.*, 54 (1982) 2134.
15 P.-O. Larsson, M. Glad, L. Hansson, M.-O. Mansson, S. Ohlson and K. Mosbach, *Adv. Chromatogr.*, 21 (1983) 41.

16 W. Becker, *Immunochem.*, 6 (1969) 539.
17 N. M. G. Harboe and A. Ingild, *Scand. J. Immunol.*, 17, Suppl. 10 (1983) 345.
18 P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk, *Anal. Biochem.*, 150 (1985) 76.
19 R. M. Moore and R. R. Walters, *J. Chromatogr.*, 317 (1984) 119.
20 R. R. Walters, *Anal. Chem.*, 55 (1983) 591.
21 U. Lund, *J. Liq. Chromatogr.*, 4 (1981) 1933.
22 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
23 G. Mancini, A. O. Carbonara and J. F. Heremans, *Immunochemistry*, 2 (1965) 235.
24 I. Björk, B.-Å. Petersson and J. Sjöquist, *Eur. J. Biochem.*, 29 (1972) 579.
25 R. R. Walters, J. F. Graham, R. M. Moore and D. J. Anderson, *Anal. Biochem.*, 140 (1984) 190.
26 R. C. Duhamel, P. H. Schur, K. Brendel and E. Meezan, *J. Immunol. Methods*, 31 (1979) 211.
27 A. F. S. A. Habeeb, in M. Z. Atassi (Editor), *Immunochemistry of Proteins*, Vol. 3, Plenum Press, New York, 1979, pp. 223–299.
28 S. Yamamoto, R. Uesugi, M. Omura and H. Hirata, *Bull. Azabu Univ., Vet. Med.*, 3 (1982) 203.
29 S. C. Crowley, unpublished results, Iowa State University, 1985.
30 J. R. Sportsman and G. S. Wilson, *Anal. Chem.*, 52 (1980) 2013.
31 J. R. Sportsman, J. D. Liddil and G. S. Wilson, *Anal. Chem.*, 55 (1983) 771.
32 S. K. Roy, D. V. Weber and W. C. McGregor, *J. Chromatogr.*, 303 (1984) 225.
33 W. U. de Alwis and G. S. Wilson, *Anal. Chem.*, 57 (1985) 2754.
34 T. M. Phillips, *LC Liq. Chromatogr., HPLC Mag.*, 3 (1985) 962.
35 T. Peters, Jr., in O. Bodansky and C. P. Stewart (Editors), *Advances in Clinical Chemistry*, Vol. 13, Academic Press, New York, 1970, pp. 37–109.
36 W. M. Hitzig and J. Jako, in H. C. Curtius and M. Roth (Editors), *Clinical Biochemistry*, Vol. 2, De Gruyter, New York, 1974, pp. 1499–1500.
37 W. Van der Loo and R. Hamers, *Protides Biol. Fluids*, 23 (1976) 603.
38 T. V. Raglione, N. Sagliano, Jr., T. R. Floyd and R. A. Hartwick, *LC Liq. Chromatogr. HPLC Mag.*, 4 (1986) 328.