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HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY: A RAPID TECHNIQUE FOR THE ISOLATION AND QUANTITATION OF IgG FROM CEREBRAL SPINAL FLUID

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SUMMARY

Standard techniques for the quantitative measurement of IgG in cerebral spinal fluid take up to 24 hs. This often delays diagnosis and treatment, critical in newborn infants. A high-performance affinity chromatography (HPAC) column, containing immobilized anti-IgG antibody, produced the same or better results in 1 h. The HPAC system gave a 98% correlation with the standard techniques at the normal–abnormal IgG level, but was more accurate at the extremely low IgG level.

INTRODUCTION

The measurement of immunoglobulins in cerebral spinal fluid (CSF) has always posed a problem due to the extremely low levels present. The major immunoglobulin, IgG, is usually measured by such standard immunological techniques as radial immunodiffusion, electroimmunodiffusion and laser nephelometry[1–3]. All of these techniques require the use of either a concentrated sample or specialized ultra-low-sensitivity immunodiffusion plates. In either case, a minimum of 24 h is required before a satisfactory result may be obtained. Radioimmunoassay is also available, but has been developed for the measurement of specific antigens, involved in demyelinating diseases, such as myelin basic protein, rather than IgG levels[4].

Affinity chromatography has been applied in many different low-pressure chromatography systems[5] but rarely as a rapid isolation system. In the past, the use of immobilized antibodies as the bioselective agents in affinity chromatography always had the disadvantage that the immobilized antibodies were not oriented properly. This problem was corrected when protein A, a bacterial cell wall protein found in Cowans Strain *Staphylococcus aureus*, was shown to have a high affinity for the Fc or tail portion of IgG antibodies[6]. Coupling protein A to various chromatographic, packing media resulted in the development of a universal affinity chromatography matrix to which IgG antibodies could be attached and correctly oriented, with the antigen receptors pointing into the fluid phase of the column[7]. In this paper we will describe a practical application of high-performance affinity chromatography

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(HPAC) with anti-human IgG antibodies immobilized on protein A coated glass beads, for the isolation and quantitation of IgG levels in unconcentrated CSF.

THEORETICAL

Immunological, affinity columns depend on the ability of immobilized antibodies to react selectively with their specific antigens:

immobilized antibody + antigen $\rightleftharpoons$ antibody–antigen complex

The antibody will select and retain specific antigens while allowing the remaining materials in the sample to pass through the column (Fig. 1A). The captured antigen is recovered by elution with acid. The hydrogen ions in the eluent interfere with the bonding between the immobilized antibody and the antigen, thus releasing the antigen (Fig. 1B). The released antigen then flows though the column and is measured by the column detector (Fig. 1C).

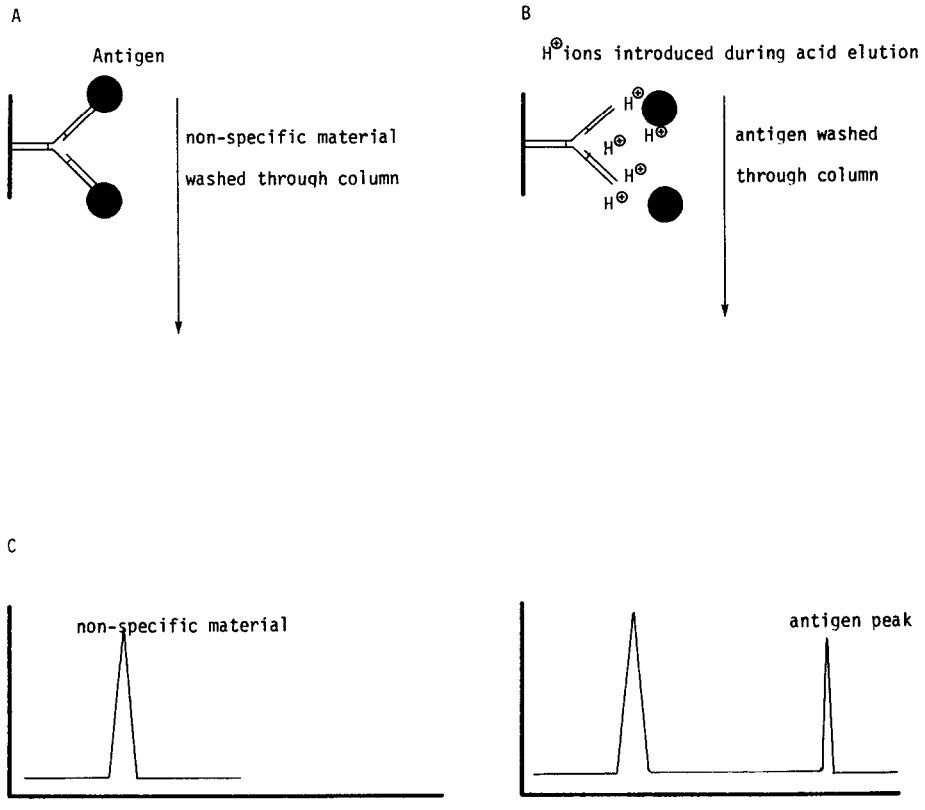

Fig. 1. Diagram of affinity chromatography with immobilized antibodies. A shows the immobilized antibody retaining the specific antigen while the unreactive material passes through the column. B shows the prianciple of alution with acid, where bound antigen is released following the disruption of the antibody–antigen bond by the hydrogen ions in the eluent. C shows the elution pattern as the chromatogram is developed from stage A to stage B.

EXPERIMENTAL

Materials

Carbonyl diimidazole glycophase-derivatized controlled-pore glass beads (Pierce, Rockford, IL, U.S.A.) were acid-washed prior to use and stored in double distilled water until required. Protein A (Pharmacia, Piscataway, NJ, U.S.A.) was obtained as a lyophilized pure product. The goat anti-human IgG heavy-chain specific antibody (Miles Biochemicals, Elkhart, IN, U.S.A.) was obtained as a purified IgG fraction and dialysed against 0.9% sodium chloride (pH 7) prior to coupling it to the protein A-coated beads. The 1-cyclohexyl-3-(2-morpholinethyl) metho-*p*-toluenesulphonate carbodiimide protein cross-linker was purchased from Sigma (St. Louis, MI). IgG standards (Capple Labs., Malvern, PA, U.S.A.) were made up at 1 mg/ml, 100 μg/ml, 10 μg/ml and 1 μg/ml in 2% human serum albumin (Cappel Labs., Malern, PA, U.S.A.), dissolved in 0.9% sodium chloride (pH 7). All columns and column fittings were obtained from Biorad Labs. (Rockville Centre, NY, U.S.A.). Supplies for the comparison study were: ultra-low radial immunodiffusion plates (Kalstad Labs., Austin, TX, U.S.A.) and nephelometric grade anti-human IgG (Beckman, Palo Alto, CA, U.S.A.).

Column construction

Protein A was reconstituted in 10 ml of 50 m*M* carbonate buffer (pH 9) by gentle swirling. The glass beads were coated with protein A by adding 10 grams of derivatized beads to 5 mg of purified protein A. The beads and protein A were placed in a 15-ml glass-stoppered tube and mixed on an overhead mixer for 18 h at 4°C. Following this incubation, the beads were throughly washed by sedimentation in 0.9% sodium chloride (pH 7). To each gram of protein A-coated beads, 5 ml of 0.9% sodium chloride containing 1 m of purified goat anti-human IgG heavy-chain specific antibody was added and the mixture was placed on the overhead mixer for 1 h. The beads were throughly washed by sedimentation in 0.9% sodium chloride, and the protein A-bound antibody was cross-linked to the protein A by incubation with 10 m*M* carbodiimide solution (pH 9). The beads in 10 ml of carbodiimide solution were placed on the overhead mixer for 1 h. The beads were again throughly washed and slurry-packed into a 15 cm × 4.6 mm I.D. stainless-steel column at 2000 p.s.i. Fig. 2 illustrates this procedure.

Chromatography

The column was connected to a Beckman 340 isocratic system, equipped with a Chromatron solvent selection–low-pressure gradient controller, a Model 160 UV detector set at 280 nm, a Model 112 pump and a Shimadzu C-RIB data processor. Samples were injected into a 210 Altex injection port with a 100-μl sample loop. Chromatography was performed by developing the column isocratically for 20 min with 0.9% sodium chloride, 0.1 *M* sodium acetate buffer (pH 6.5) at a flow-rate of 0.5 ml/minute. After the initial isocratic elution, during which the material other than IgG was washed out of the column, a pH gradient from pH 6.5 to 1.0 was formed over 10 min by the addition of 0.1 *N* hydrochloric acid. This concentration was maintained for another 20 min, during which time the antigen was released and passed through the column. At the end of each analysis, the column was recycled with the original buffer. Fig. 3 is a flow-chart of the chromatography procedure.

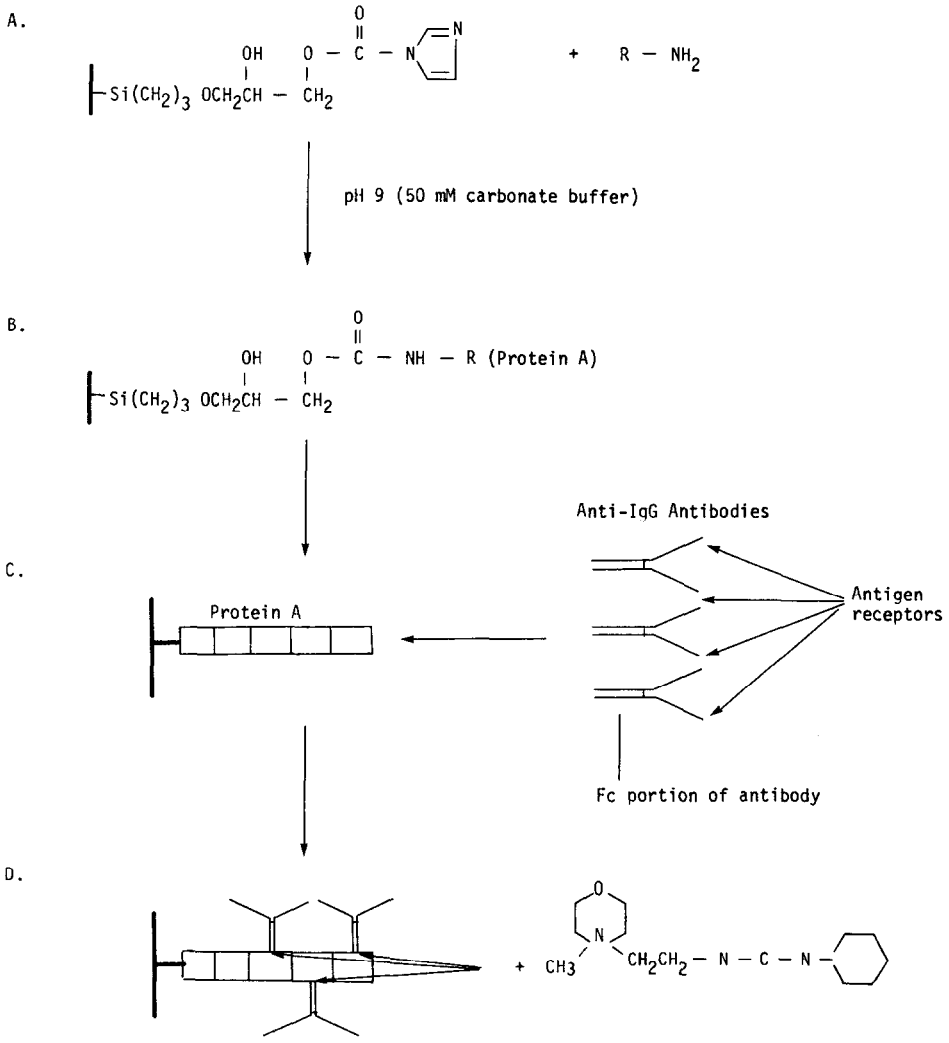

Fig. 2. Chemical basis of the column preparation. A and B show the coupling procedure for coating the glass beads with protein A. C and D illustrate the binding of antibodies to protein A and their cross-linking with carbodiimide.

Comparative immunological testing

The analysis of CSF samples and IgG standards by HPAC was compared with radial immunodiffusion in ultra-low IgG plates and by rate nephelometry in a Beckman Immunochemistry System (ICS). All standards were dissolved in a 2% human serum albumin solution, as albumin constitutes the major protein of CSF. Both radial immunodiffusion and nephelometry were performed according to the manufacturers' instructions. CSF samples for rate nephelometry were concentrated ten-fold in a negative pressure dialysis system prior to testing.

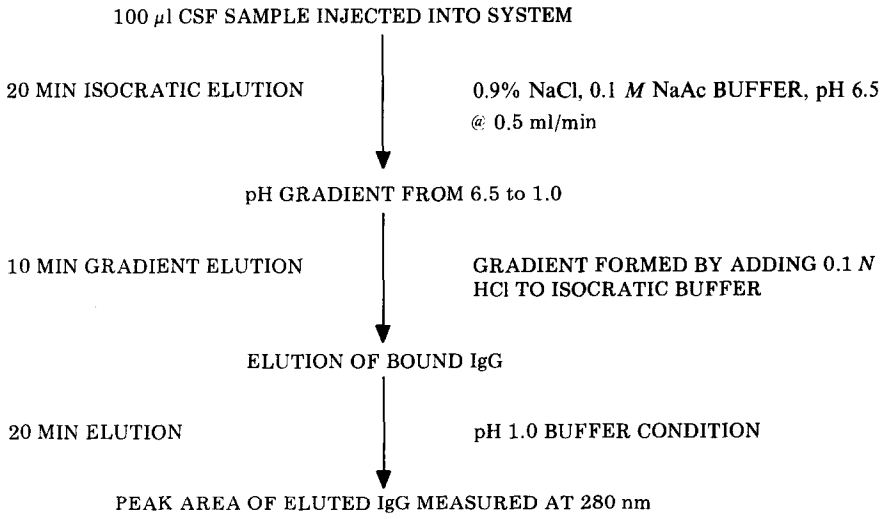

Fig. 3. Flow-Chart of the HPAC procedure.

RESULTS AND DISCUSSION

A rapid technique for the quantitation of IgG in CSF samples by HPAC has been developed. Typical chromatograms produced by the HPAC method are shown in Fig. 4. Fig. 4A is the profile produced by passing a 100-μg/ml IgG standard, dissolved in human serum albumin solution, through the system. The chromatogram shows two distinct peaks, corresponding to albumin and IgG, respectively. The chromatogram shown in Fig. 4B demonstrates that the same resolving power can be maintained when a normal sample of CSF is processed. In this case, the albumin peak is less well defined, but is still quite distinct from the IgG peak. However, when an abnormally high IgG CSF sample from a patient with viral encephelitis is processed, the increased IgG peak is clearly defined.

Comparison of results on IgG standards between HPAC and radial immunodiffusion and rate nephelometry showed that all three techniques gave similar results, the HPAC technique being as sensitive as the nephelometric assay yet producing the results in a fraction of the time (Table I). However, when patient samples were compared, all three of the assays gave similar results but the HPAC system was more accurate in the ultra-low IgG ranges. In the twenty patient samples that have been analyzed to-date, this correlation has remained unchanged.

IgG makes up 10% of normal CSF protein and is usually derived from the serum[8] but elevated levels of IgG are considered to be of great diagnostic significance in multiple sclerosis, neurosyphilis, subacute sclerosing panencephalitis, allergic encephalitis, amyotrophic sclerosis, and in the chronic phase of central nervous system infections[8–10]. The ratio of CSF IgG–total protein provides data which help to assess whether local IgG synthesis is taking place or whether increased permeability of the blood-brain barrier is present[10]. The measurement of the non-IgG peak in the HPAC chromatogram together with the measurement of the IgG peak gives the total protein

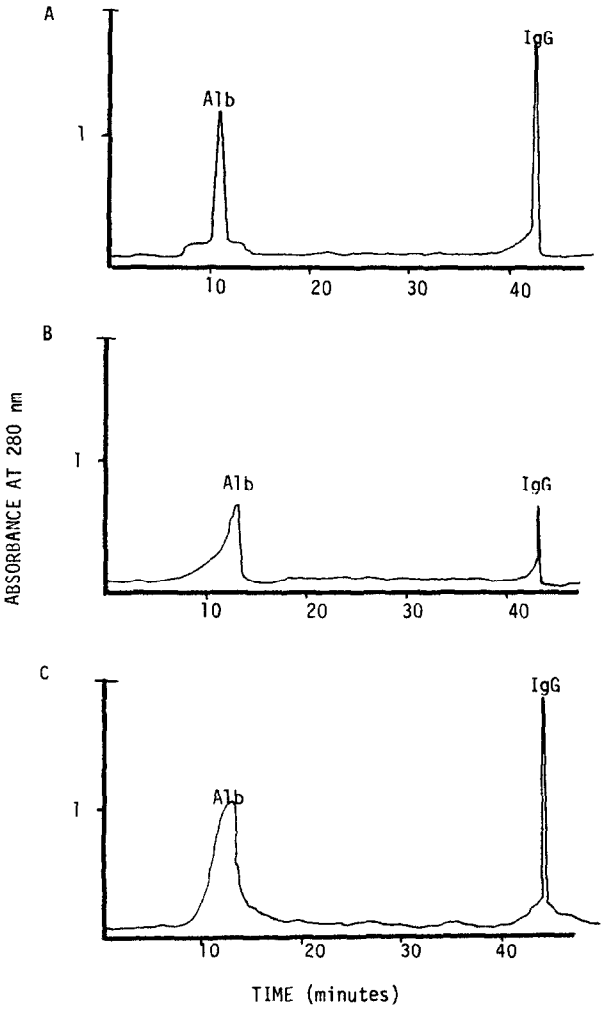

Fig. 4. Typical chromatograms produced by HPAC. A demonstrates the separation of serum albumin from IgG standard. B shows the pattern produced by a normal CSF sample and C illustrates the chromatogram produced by a CSF sample which has abnormally high IgG.

content of the sample and this can be compared with the IgG peak to give the IgG–total protein ratio without further manipulation of the sample. This is an advantage of the HPAC system over either the radial immunodiffusion or rate nephelometry assays, where total protein is estimated by performing additional analysis of the sample.

In addition, the development of the protein A-coated glass beads provides a universal immobilization media for any IgG antibody. Most mouse IgG antibodies are bound by protein A, and this makes this matrix particularly suitable for immobilizing any of the vast array of monoclonal antibodies available today. The structure and composition of the glass beads is such that the columns will withstand the rigors

TABLE II

COMPARISON OF HPAC WITH STANDARD IMMUNOLOGICAL TECHNIQUES

All values expressed in μg/ml.

Sample	*HPAC*	*RID**	*Nephelometry*
IgG standards			
1 mg/ml	1002	995	1002
100 μg/ml	98	92	101
10 μg/ml	8	< 10	7
1 μg/ml	1.3	< 10	2
CSF samples			
Normal CSF	38	35	37
High/abnormal CSF	93	91	94
Low/abnormal CSF	12	14	12

* Radial immunodiffusion.

of high performance chromatography. This makes this technique particularly attractive as a fast, quantitative method for isolating specific antigens by immunoselectivity.

CONCLUSIONS

HPAC is a method for fast isolation and quantitation of specific biological materials. The use of protein A-coated glass beads provides a simple matrix for the immobilization of any IgG antibody, especially the growing number of available monoclonal antibodies. HPAC can be performed on any high-performance liquid chromatograph and adds the power of immunological specificity to any biological isolation procedure.

The use of HPAC for the isolation and quantitation of CSF IgG is just one application but an important one because the method is as accurate as the standard immunological assays used in most clinical laboratories yet it is faster and yields results on 100 μl of unconcentrated sample, which is a valuable asset in pediatric medicine.

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