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Flow injection immunoassay using a protein A immunoreactor*

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Abstract: Competitive immunoassays have been developed for the immunosuppressant cyclosporin A and the anti-asthmatic drug theophylline utilizing identical controlled pore glass–protein A microcolumns and flow injection techniques. For cyclosporin A the assay was based on a monoclonal antibody with fluorescence detection whilst for theophylline sheep antiserum and electrochemical detection was used.

Keywords: *Cyclosporin A; theophylline; flow injection immunoassay; protein A; fluorescence detection; electrochemical detection.*

Introduction

Protein A is a protein component found in the cell wall of more than 90% of strains of *Staphylococcus aureus* which binds the F_c region of many immunoglobulins of most mammalian species [1]. It is isolated from the cell wall as a highly stable 42 000 molecular weight protein which retains its activity after exposure to 4 M urea, 4 M thiocyanate, 6 M guanidine hydrochloride and extremes of pH and temperature [2]. Although first described in 1940 [3] it was not until the mid 1960s [4] that it was first demonstrated that the binding between protein A and antibody occurred at the F_c region and not at the antigen binding F_{ab} fragment. This realization enabled workers to exploit protein A as an antibody binding protein in immunochemistry and immunoassays. The aim of this work was to produce an immunoassay employing protein A immobilized on a solid phase and incorporating flow injection techniques.

Currently most solid-phase immunoassays are performed with 96 well microtitre plates in which samples can be processed simultaneously. This technique has proven to be sensitive, but it is only semiquantitative and is difficult to automate [5, 6]. Flow injection analysis (FIA) on the other hand is an easily automated technique that can be adapted to accommodate many immunoassay formats. Previously developed FIA immunoassays use electrochemical [7], or optical detection [8, 9]. FIA is an attractive technique to apply to immunoassays because precise control of reagent addition and reaction times offers the potential for high analytical precision.

Sepharose [10], non-porous silica [9, 11], Trisacryl GF 2000 [12], Pall Immunodyne membrane [13] and Biomag 4100 beads [13] have all been used as solid phases in immunoreactors in flow injection immunoassays. Controlled pore glass exhibits physical properties which make it ideal as a solid support for flow injection immunoassays and displays none of the limitations usually associated with soft gels such as agarose or Sepharose, i.e. compression and attrition of gel in a flowing system [14]. The unique features of controlled pore glass are high mechanical strength, high flow rate capability (non compressible), high stability in solvents and acids, thermally stable, stable bead size in changing environments and low non-specific protein adsorption.

A number of solid phase immunoassays have been developed employing a range of binders; a binder being any molecule which exhibits molecular recognition for another molecule (ligand). Antibodies are the binders most commonly employed in ligand binder assays; both polyclonal and monoclonal antibodies have been used. Other binders include cell-surface receptors [15], carrier or transport

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proteins, such as thyroxine binding globulin [16], riboflavin binding protein [17], avidin [18], concanavalin A [19], protein A [20], DNA [21] and protein G [22].

Antibodies are by far the most common ligand binders used in solid phase assays that utilize flow injection techniques [9, 23], however the antibodies are coupled to the support matrix in a random fashion limiting the antigen binding capacity, although attempts have been made to overcome this problem [7]. In addition the specificity of the immobilized antibody means that only a very limited range of antigens can be bound by each immunoaffinity column. These problems can be largely overcome by the use of protein A, most commonly immobilized on Sepharose [24] and controlled pore glass [25]. Because protein A binds the F_c region of antibodies the antigen receptors are orientated away from the support material and into the mobile phase maximizing potential binding sites. In addition a whole range of antibodies with different specificities can be bound to and eluted from the affinity column [1] ensuring that the matrix not only has high antibody binding efficiency but is also extremely flexible in its use.

A number of methods have been developed employing immobilized protein A in a flowing system, the majority of which are used for isolating and purifying antibodies [24, 26] and labelled antibody conjugates [27]. Surprisingly the use of protein A in flow injection immunoassays appears limited [10].

The extreme flexibility of use of protein A has enabled this laboratory to develop competitive immunoassays for two therapeutic drugs using a controlled pore glass–protein A microcolumn and flow injection techniques with either a monoclonal antibody and fluorescence detection or polyclonal antiserum and electrochemical detection with enzymic amplification.

Experimental

Materials and methods

All cyclosporin congeners and the parent compound specific monoclonal antibodies were kind gifts from Sandoz (Basle, Switzerland). Tetramethyl-rhodamine-cadaverine (TRC) was purchased from Molecular Probes (Eugene, OR, USA), MeOH was HPLC grade (Fisons, Loughborough, Leicestershire, UK), plastic centrifuge tubes (73 × 23 mm Luckham Ltd, West Sussex, UK) were used for incubations. Controlled pore glass–Protein A (CPG–ProA) was purchased from Oros Instruments (Slough, UK). Glass microcolumns (50 mm × 3 mm i.d.) were obtained from Omnifit Ltd (Cambridge, UK) and were packed with CPG–ProA using a peristaltic pump. For the fluorescence method PBS/BSA, pH 7.4 was used as the assay dilution buffer with the following composition: 40 mM Na_2HPO_4; 8 mM NaH_2PO_4; 150 mM NaCl; 0.01% BSA and 0.1% NaN_3. Antibody was prepared in assay buffer at the appropriate concentration. Cyclosporin standards were prepared in assay buffer by diluting 1:100 from methanolic stock solutions of CyC at appropriate concentrations. The cyclosporin tracer (Cy-TRC) was prepared from [(*O*-succinimidooxysuccinyl)-Thr]^2 cyclosporine and tetramethyl rhodamine cadaverine according to the literature [28].

Theophylline was obtained from the Sigma Chemical Company (Poole, Dorset, UK). Theophylline-8-butyric acid lactam was purchased from the Novabiochem Chemical Company (Nottingham, UK). *p*-Aminophenyl phosphate was synthesized from *p*-nitrophenyl phosphate as previously described [29].

Immunoassay grade alkaline phosphatase (grade 1 from calf intestine 10 mg ml^{-1} and >2500 U mg^{-1}) was obtained from Boehringer Mannheim (Lewes, East Sussex, UK). Sheep anti-theophylline antisera was purchased from International Laboratory Services (ILS) (London, UK). The theophylline–alkaline phosphatase conjugate was prepared according to a published procedure [30]. All other reagents were of (AnalaR) grade and all solutions were prepared in water purified by the Liquipure Modulab system. For both methods the Tris equilibration and citrate elution buffers were prepared according to the Oros data sheet except that 0.5 M NaCl was added to the citrate buffer.

Instrumentation

Apparatus for flow injection analysis was as follows: the flow of the eluent or carrier stream was produced with an LKB 2132 Microperpex peristaltic pump. Injections of solutions were made with a Rheodyne 5020 injection valve fitted with a 100 μl loop for the fluorescence method and a 25 μl loop for the electrochemical method. All fluorescence measurements were made at room temperature on a Perkin–

Elmer LS-50 fluorescence spectrometer interfaced to an Epson AX-3 personal computer. The eluent was monitored using a 100 μl flow cell from Hellma (Essex, UK) positioned in the sample chamber of the instrument. Excitation and emission monochromators were fixed at 550 and 580 nm, respectively.

For the electrochemical method a pulse damper constructed from glass with a platinum wire ground connection, was fitted between the perstaltic pump and the injection valve to eliminate the static electricity pulses generated by the peristaltic pump. A wall jet detector cell containing the platinum working electrode, the stainless steel counter electrode and the saturated calomel reference electrode was housed in a metal box. The potential of the platinum electrode was controlled by means of a Dionex Ionochrom Pulsed Amperometric detector.

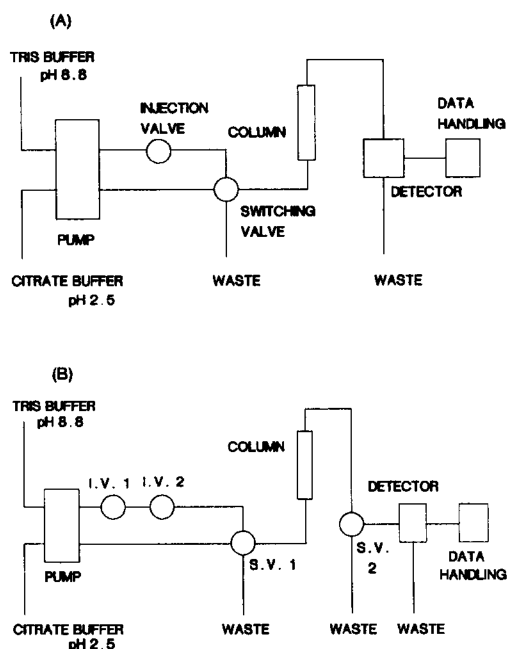

Figure 1
Schematic representation of the flow injection manifolds for the immunoassay of (A) Cyclosporin A with fluorescence detection. (B) Theophylline with electrochemical detection.

Fluorescence immunoassay procedure

The cyclosporin tracer was prepared in MeOH at the appropriate concentration and volume and 20 μl aliquots transferred to incubation tubes. The solvent was then removed by evaporation under reduced pressure and the tubes stored in the dark at room temperature in a dessicator cabinet. Each sample and standard was treated in the same way. The tracer was reconstituted into 150 μl of assay buffer or standard and left for 10 min, after which 50 μl of antibody solution was added and the mixture allowed to incubate for 10 min. The mixture was then injected onto the column which had been equilibrated with Tris buffer (Fig. 1A). After 400 s the flow through the column was switched to citric acid pH 2.5 to elute the bound sample and switched back to Tris after 700 s. Binding and elution flow rates were maintained at 0.5 ml min^{-1}. The elution peak area was determined using the LS-50 Fluorescence Data Manager software. It was found that the column required 10 min equilibration with Tris buffer to prevent tailing of the unbound label when the next sample was injected. The total assay and equilibration time, from injection to injection was 22 min.

Electrochemical enzyme immunoassay procedure

Figure 1(B) shows a schematic diagram of the system used. The typical system used for theophylline was as follows; a known amount of enzyme labelled theophylline (50 μl) and a given amount of standard free analyte (100 μl in Tris equilibration buffer) were mixed with 50 μl of the theophylline antisera (1:100 dilution in 0.15 M PBS pH 7.2). The mixture was incubated for 10 min and loaded into the sample loop of injection valve 1 and introduced onto the CPG-protein A microcolumn using the Tris equilibration buffer at a flow rate of 0.4 ml min^{-1}. At the same time switching valve 2 was directed to waste to prevent unbound protein from fouling the platinum working electrode. The actual exposure time for this mixture within the microcolumn was very short (*ca* 20 s), after which the carrier buffer washed off the unbound species for a few minutes. When injection valve 2 was opened the substrate solution (*p*-aminophenyl phosphate) was passed through the column at a flow rate of 0.6 ml min^{-1}. The product of the enzymic reaction was measured downstream by the electrochemical cell in a wall jet configuration. Results (i.e. the oxidative peak areas of *p*-aminophenol) were recorded on a Spectra-Physics SP4290 integrator. The system was regenerated by washing with citrate elution buffer for 2 min to dissociate the complex between the theophylline antisera and the immobilised protein A. The microcolumn was then equilibrated for 2 min with the Tris

buffer, after which the system was ready for another sample. The total time of the assay including the regeneration and re-equilibration steps was 18 min.

Calculation of results

Dose–response curves were prepared by plotting $(B/B_0)\%$ vs the drug concentration in the standards

$$(B/B_0)\% = \frac{\text{Peak area at stated drug conc.}}{\text{Peak area at zero drug conc.}} \times 100. \quad (1)$$

Results and Discussion

The flexibility of protein A and its use in a microcolumn has enabled the development of flow injection immunoassays for the therapeutic drugs Cyclosporin A and theophylline. Protein A has species dependent binding to immunoglobulins [1], however, assays have been developed using mouse monoclonal antibodies and sheep antiserum which show similar binding properties to protein A under identical conditions. Typical binding and elution profiles for the fluorescence method is shown in Fig. 2. Figure 3 illustrates the interaction between immobilized protein A and mouse monoclonal antibody and sheep antiserum by monitoring the amount of antibody capture for each species at increasing binding flow rates. The amount of antibody captured was determined for the fluorescence method by monitoring at the native antibody fluorescence wavelengths. A fixed amount of monoclonal antibody was injected onto the column and eluted as per assay protocol and the area of the elution peak was determined. This was repeated at varying flow rates. For the electrochemical method the antiserum was injected onto the column at varying flow rates followed in turn by fixed quantities of the enzyme labelled theophylline and substrate at constant flow rates. The product was monitored as per assay protocol, and the peak area can be considered to indicate the quantity of antibody bound to the column. For both species the binding of immunoglobulin to protein A followed a similar pattern for a fixed set of analytical conditions illustrating the flexibility of use of the controlled pore glass–protein A microcolumn.

Cyclosporin A and theophylline have different therapeutic ranges, for cyclosporin A monitoring is in the concentration range 10–1500 ng ml^{-1} whilst for theophylline it is necessary to monitor between 8 and 20 μg ml^{-1}. In this work we have developed assays for these drugs using identical controlled pore glass–protein A microcolumns, calibration curves for each assay are shown in Fig. 4. These illustrate the potential for selecting the

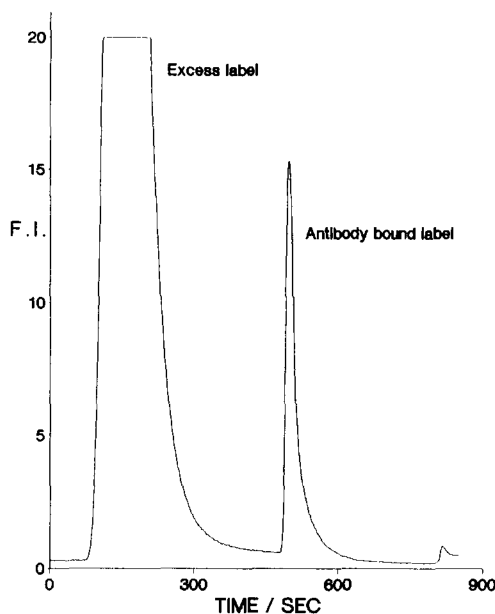

Figure 2
Typical binding and elution profile for the fluorescence method.

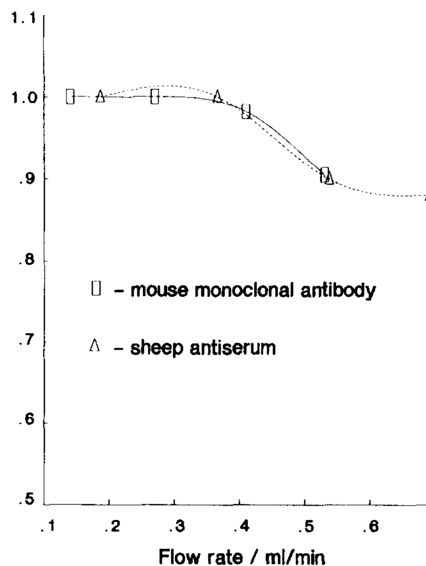

Figure 3
Normalized immunoglobulin binding to the controlled pore glass–protein A at different flow rates.

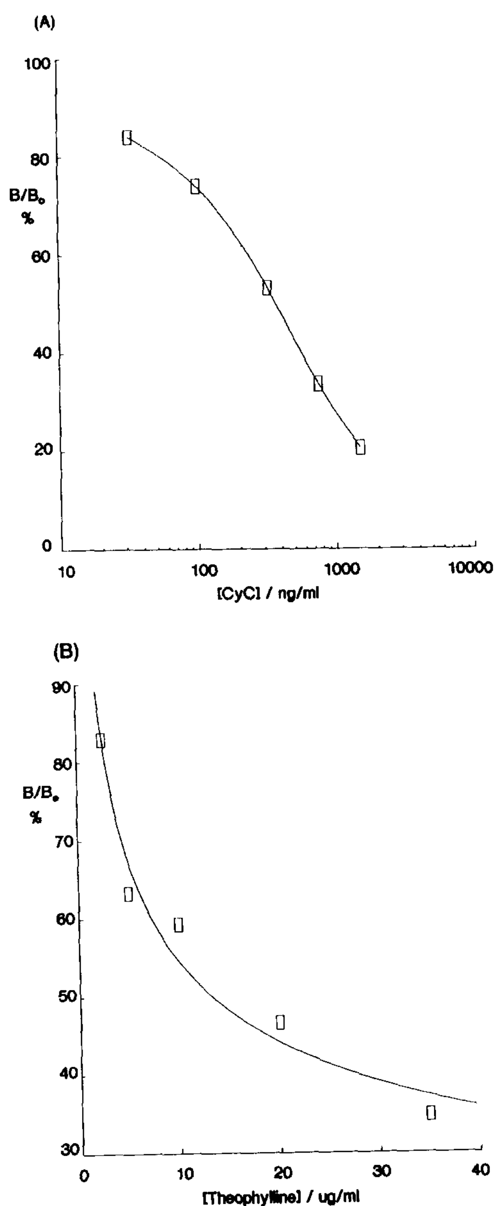

Figure 4
Calibration curves for (A) Cyclosporin A, (B) theophylline.

required assay range over several orders of magnitude.

Initial work has shown good assay precision with a RSD of 5.6% at 132.5 ng ml^{-1} and 6.0% at 532.5 ng ml^{-1} for the cyclosporin assay and 5.4% at 10 μg ml^{-1} and 4.6% at 35 μg ml^{-1} for the theophylline assay. The detection limits of the cyclosporin and theophylline assays were found to be 9 ng ml^{-1} and less than 2.5 μg ml^{-1}, respectively. In both assays the lifetime of the immunoreactor was between 70 and 100 runs.

The excellent properties of the controlled pore glass–protein A matrix enable the use of high flow rates with good antibody capture. These benefits and no requirement for separate washing steps results in an improvement in assay speed compared to microtitre based immunoassays.

This work has shown that the specificity of antibodies, the range of IgG bound by protein A and the benefits of flow injection techniques should lead to the development of simple, rapid assays for a variety of analytes with the potential for automation.

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