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An on-line immunoassay method for theophylline using a protein A immunoreactor*

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Abstract: A heterogeneous fluorescence immunoassay for theophylline has been automated using a flow injection analysis system containing a protein A solid phase reactor to separate antibody-bound and unbound fluorescein–theophylline. For each sample the antibody–protein A reaction takes place at near neutral pH, and the complexes are eluted at acid pH. The antibody-binding capacity of the reaction greatly exceeds the antibody level in each sample incubation mixture, and a single reactor can be repeatedly cycled between neutral and acid pHs. Experimental variations such as reactor size, flow rate, pH values, and reactant concentrations have been studied. Theophylline could readily be determined at the μg ml^{-1} level with on-line incubation with antibodies.

Keywords: *Theophylline; flow injection analysis; heterogeneous fluorescence immunoassay; protein A reactor.*

Introduction

Flow injection analysis (FIA) methods have been utilized with a wide variety of reaction schemes, and applications in biochemistry feature strongly in their extensive literature. FIA manifolds incorporating immobilized enzymes have proved particularly popular [1] despite the poor stability of some enzymes after attachment to solid phases. FIA-immunoassays have been described much less frequently. The first such application, developed in the authors' laboratory, was a homogeneous (i.e. separation-free) fluorescence immunoassay for albumin based on energy transfer principles [2]. Kelly and Christian [3] automated a homogeneous enzyme immunoassay which utilized fluorimetric detection, and Worsfold and Hughes [4, 5] studied model immunoprecipitation assays in a flow injection system with turbidimetric detection. Several research groups have described heterogeneous flow injection immunoassays utilizing solid phase reactors. De Alwis and Wilson [6] described a sandwich enzyme linked immunosorbent assay for bovine immunoglobulin G (IgG) in which this protein was immobilized on an affinity chromatography matrix. In a later paper, the same authors immobilized the Fab' (i.e. antigen-binding) fragment of goat anti-human IgG antibodies, and thus developed a heterogeneous electrochemical immunoassay for IgG, based on the detection of IgG-conjugated glucose oxidase reaction products [7].

Locascio-Brown *et al.* [8] immobilized anti-theophylline antibodies on silica particles in an ingenious heterogeneous immunoassay whose detection procedure involved the use of fluorophore-loaded liposomes. Miller *et al.* [9] described the use of solid phase reactors in the reduction of the endogenous fluorescence of blood serum prior to fluorescence immunoassays, and Miller [10] demonstrated the use of immobilized protein A in a heterogeneous fluorescence immunoassay for human serum albumin. Protein A is a bacterial protein which binds to antibodies at a site distinct from the antigen-binding domains: a similar protein, protein G, was utilized by Janis and Regnier [11] in a complex on-line immunoassay for serum transferrin in which transferrin–anti-transferrin complexes were desorbed from a protein G reactor, dissociated, and separated on a reversed-phase chromatography column. These heterogeneous assays have in common the need to pass two different eluents through the solid phase reactors, a neutral buffer in

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which antigen–antibody or antibody–protein A/G reactions occur, and an acid buffer or a solution of a chaotropic reagent to dissociate the non-covalent complexes prior to injection of the next sample.

The present paper describes the use of a protein A reactor in a simple heterogeneous fluorescence immunoassay for theophylline. The method incorporates on-line incubation, and antibody–protein A complexes are eluted by a pH change. The applicability of the method for determining theophylline in serum is demonstrated.

Experimental

All experiments used a single-channel flow injection analysis system incorporating an Ismatec peristaltic pump, Teflon flow tubing (0.8 mm i.d.) and two Omnifit 6-way injection valves in series (Fig. 1). The protein A reactor (50 mm long, 3 mm i.d.) contained protein A (5.5 mg per ml of packed beads) immobilized on controlled pore glass particles (Oros Instruments, 75–125 μm diameter). The binding capacity of the immobilized protein A for mouse immunoglobulin G was 40–45 mg per ml of packed beads. The fluorescence detector was a Perkin–Elmer LS-5B spectrometer interfaced to a Model 3600 Data Station. The spectrometer was fitted with a 25 μl silica flow cell (Hellma) and operated at room temperature without spectral correction. Fluorescein isothiocyanate-(FITC-)theophylline, and sheep anti-theophylline antiserum were obtained from International Laboratory Services. The carrier solution was 50 mM tris-HCl buffer (pH 8.8) containing 0.1 M NaCl, and the protein A–antibody complexes were eluted with citrate buffer (0.5 M, pH 3.5) also containing 0.1 M NaCl. All solutions were prepared in de-ionized water and degassed with helium for 5 min before use. Each immunoassay was performed by injection of 50 μl of antiserum (1:100 dilution) using valve C (Fig. 1) into the tris buffer carrier. This was followed by 50 μl of an equivolume mixture of theophylline-containing sample and FITC-theophylline. Some fluorescence was detected after sample injection (excitation and emission wavelengths of 495 and 525 nm, respectively): this peak corresponded to the unbound FITC-theophylline. When elution of this unbound material was complete (<5 min), 30 μl of citrate buffer were injected through valve D (separate valves were used to avoid the possibility of samples and antibodies being contaminated with the acid buffer before the immunoreaction). This eluted the FITC-theophylline which had bound to the protein A-bound antibody. The intensity of this second fluorescence peak was measured, and used to calculate sample theophylline levels by comparison with standards. Finally, using switching valve A, the reactor was cleared for 5 min with further citrate buffer. Using this protocol each assay took not more than 20 min in all.

Results

Experimental variables

The effects of altering the carrier flow rate during the binding and elution stages of the assay were studied over the range 0.1–0.8 ml min^{-1} some loss of (bound) fluorescence intensity occurred, presumably because of a shorter reaction time between the theophylline and the immobilized antibodies: a flow rate of 0.2 ml min^{-1} was thus used throughout.

The buffer compositions most suitable for the binding and elution steps were also studied. The pH 8.8 buffer was the most effective of several used in the binding step. The citrate buffer, pH 3.5 successfully eluted bound theophylline from the reactor: it had a quenching effect on the fluorescence of the FITC-theophylline, but the residual fluorescence provided sufficient sensitivity in this assay.

Antiserum dilutions were tested over the range 1:10–1:10,000. Lower antibody concen-

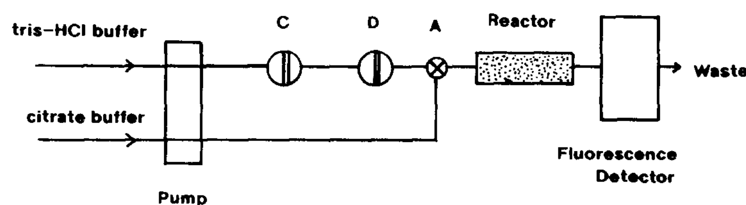

Figure 1
Flow injection manifold.

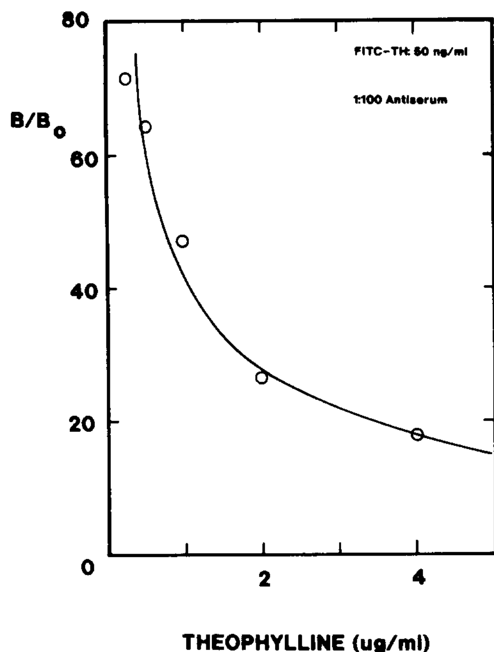

Figure 2
Calibration curve for theophylline immunoassay. The B/B_0 values are percentages.

trations than these in 1:100 diluted antiserum provided insufficient sensitivity, but higher concentrations did not improve theophylline binding in the dynamic flowing system. Figure 2 shows a typical immunoassay calibration curve, in which the assay response is expressed as B/B_0, the level of antibody-bound labelled theophylline expressed as a fraction of the bound labelled material at zero theophylline concentration. As expected in a heterogeneous assay, fluorescence signals were very similar in serum and in pure theophylline solution: in practice serum samples were diluted 1:10 before study to minimize blockage of the frits in the protein A reactor. The within-day and between-day precisions of the assay, tested with 10 replicates in each case, were similar at 7.9% RSD. Recoveries of theophylline from spiked serum were 94.8, 92.1 and 80.5% at 0.25, 1.0 and 2.0 $\mu g\ ml^{-1}$, respectively.

A single protein A reactor, used as described above, could be employed for hundreds of assays lasting over 2–3 weeks before needing replacement because of diminishing antibody binding activity.

Discussion

The assay described in this paper permits the determination of serum theophylline levels in a simple manner. The value of the method lies in its generality: protein A reactors can be used to determine a variety of analytes by flow injection immunoassay, and with a variety of detection systems, including electrochemical detection of an enzyme label. Economy of sample and reagent are other advantages of the method. An important feature of this approach to immunoassay is that the protein A columns have a binding capacity for antibodies which greatly exceeds the quantity of antibody used in each assay. As a result, loss of binding capacity by the reactor has to be large before the reactor is no longer usable.

Two improvements to the assay are under active development. One involves the use of the fluorescent label Lucifer Yellow VS [12], whose emission intensity is much less pH dependent than that of fluorescein. A second modification, which will increase the sampling rate of the assay, consists of the inclusion in the flow injection manifold of two protein A columns in parallel: one column is flushed with acid buffer while the second is being used for the binding reaction. In this way, a simple on-line flow injection immunoassay system, widely applicable and with a good sampling rate, can be developed.

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