Enzyme-Linked Flow-Injection Immunoassay Using Immobilized Secondary Antibodies

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Abstract. A fast, non-equilibrium enzyme-linked flow-injection immunoassay (FIIA) system using an immobilized secondary-antibody reactor is described. The assay method is based on the competition between the enzyme-labeled antigen and analyte (unlabeled antigen) for a limited amount of soluble primary-antibody binding sites. This mixture is then introduced via flow-injection into the secondary-antibody reactor. The reactor bound enzyme activity, as measured by flowing an appropriate substrate solution through the reactor, is inversely proportional to the concentration of free analyte in the sample. By using non-equilibrium conditions, a single assay takes a total time of 15 min or less including regeneration of the reactor. To illustrate the application of this system, theophylline and insulin were chosen as model hapten and macromolecule analytes, respectively. Preliminary studies suggest that the new FIIA system is suitable for determining theophylline in serum with acceptable accuracy and precision.

Key words: theophylline, insulin, competitive binding assays, flow injection analysis, ion-selective electrode.

In recent years, newer enzyme-immunoassay (EIA) methods have essentially replaced classical radioimmunoassay (RIA) procedures for the detection of a wide variety of biomolecules (e.g., proteins, drugs, hormones, etc.) at trace levels [1—4]. Modern EIA methods can be grouped into two classes: homogeneous and heterogeneous. The more widely used heterogeneous (or solid-phase) procedures are often based on the competition between the analyte and enzyme-labeled analyte for a limited number of primary(1st)-antibody binding sites (antibody toward analyte) immobilized on solid particles. After a lengthy equilibration period and subsequent separation and washing steps, the enzymatic activity bound to the solid phase is determined by adding the substrate(s) for the enzyme. Bound enzymatic activity is inversely related to concentration of analyte in the sample. At

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present, solid-phase EIA methods based on this protocol are quite time consuming and involve many manual pipetting and washing steps which increase the potential for error, and hamper efforts to fully automate such methods. We now report on the use of a novel flow-injection arrangement in conjunction with an immobilized secondary(2nd)-antibody reactor which can be used to simplify and speed-up heterogeneous EIA procedures.

The development of EIA arrangements involving flow-through immunosorbents and a variety of downstream detectors has been reported previously [5, 6]. In most cases, the 1st antibody has been immobilized on solid particles (e.g., silica) and packed into a small flow-through reactor. Preparation of such 1st-antibody reactors requires careful control of the coupling conditions and amount of 1st-antibody actually linked to the packing material. This is because the 1st-antibody is typically the limiting reagent in these assays and the immobilized binding site concentration dictates the ultimate detection limits of the method. Random orientation of the immobilized 1st-antibody molecules can also cause heterogeneity in binding affinities (including lower affinity) toward analyte and enzyme-labeled analyte. In addition, for repetitive use, such columns must be continually regenerated by dissociating the bound analyte and enzyme-labeled analyte conjugates after each assay. These regeneration steps (usually pH ≤ 4.0) may result in loss of specific analyte binding ability, and with time, this can seriously affect the observed dose-response behavior of the system (binding less enzyme-labeled analyte). Such antibody denaturation has been observed previously with immunosensors based on immobilized primary antibodies [7].
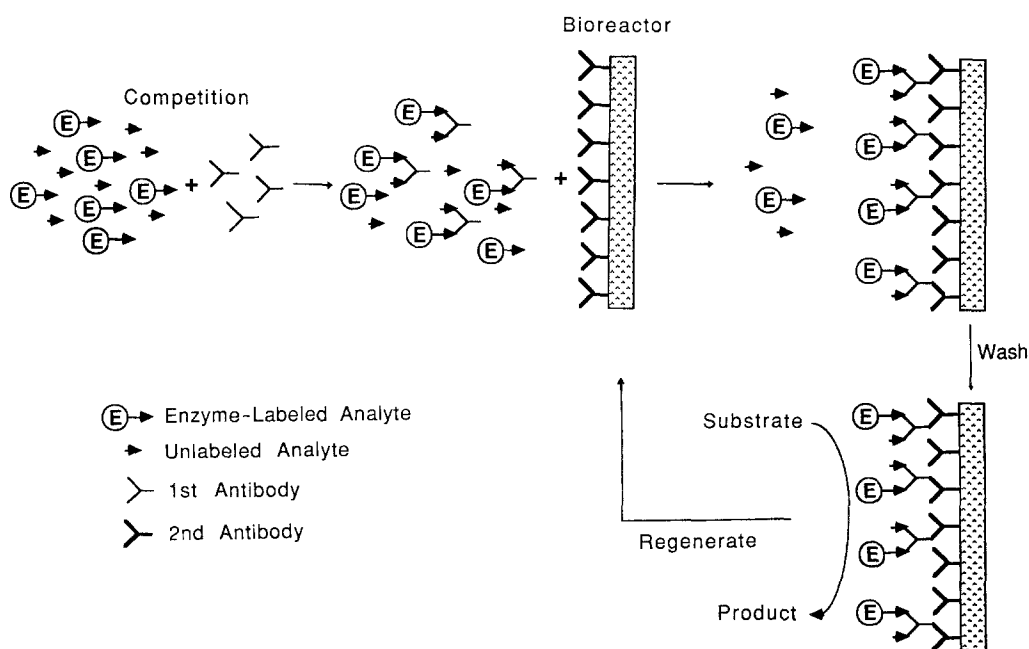

Fig. 1. Schematic diagram illustrating the principles of the solid-phase enzyme-linked FIIA system

In this report, we describe a new flow-injection immunoassay system (FIIA) which makes use of immobilized 2nd-antibodies as a means of separating bound and free enzyme-labeled analyte in a flowing sample solution. Fig. 1 illustrates the principles of the proposed system. The sample containing the analyte is first equilibrated with given amounts of enzyme-analyte conjugate and 1st-antibody. Since the 1st-antibody is added as a soluble reagent, its site concentration can be well controlled, and its natural affinity and homogeneity is preserved. After a brief non-equilibrium incubation period, the reaction mixture is injected through the immunosorbent reactor containing excess 2nd-antibody (antibody which selectively binds the 1st-antibody). A fixed fraction of the total 1st-antibodies binds to the column (non-equilibrium amounts) with associated analyte or enzyme-labeled analyte. All unbound species are washed away by the carrier stream buffer. Enzyme activity bound to the reactor can be detected by diverting a flowing solution of substrate through the reactor, and monitoring the product downstream with an appropriate flow-through electrochemical or photometric detector. After detecting bound activity, the reactor is regenerated by diverting a low pH buffer through the column for brief period. As shown in Fig. 2, the flow of various solutions through the system is controlled by several rotary injection valves.

To demonstrate the FIIA concept described above, we have chosen theophylline and insulin as model analytes. In the case of theophylline, adenosine deaminase (ADA) is used as the labeling enzyme, and its activity is detected downstream with an ammonium ion-selective electrode. For insulin, horseradish peroxidase (HRP) is employed as the label with photometric detection of bound enzyme activity. Sheep anti-theophylline and guinea pig anti-insulin are utilized as 1st-antibodies while the reactors
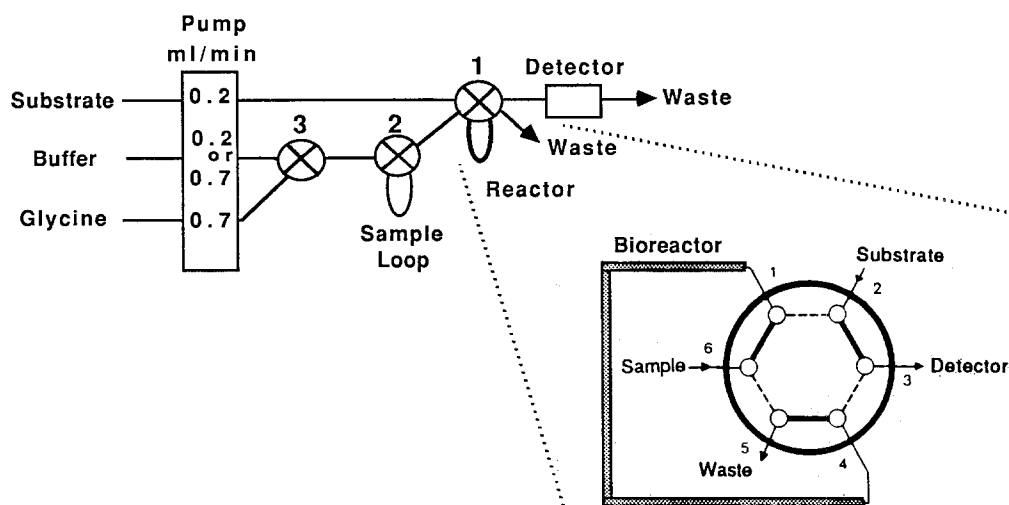

Fig. 2. Flow and valve arrangement used in 2nd-antibody reactor based FIIA system. Flow rate of buffer stream controls contact time between 2nd-antibody reactor and 1st-antibody reaction mixture injected via rotary valve 2. Buffer flow rate was 0.7 ml/min for insulin system and 0.2 ml/min for most theophylline measurements

contain immobilized anti-sheep whole serum and anti-guinea pig whole serum, respectively. Using these reagents, reactors, and the flow configuration illustrated in Fig. 2, it will be shown that very rapid (e.g., 10—15 min) and selective heterogeneous enzyme-linked methods for detection of small haptens (e.g., theophylline) and larger proteins (e.g., insulin) can be developed.

Experimental

Apparatus

The flow system used for the FIIA measurements is shown in Fig. 2. A Rainin Rabbit peristaltic pump was used to deliver the sample and buffers through the system. Potentiometric measurement of the ammonium ions produced from the adenosine deaminase (ADA) catalyzed reaction was made using a tubular flow-through ammonium ion-selective electrode similar in design to that described previously [8, 9]. The potential readings were taken on an Altex SelectIon 2000 pH/mV meter and recorded on a Linear Model 1201 strip-chart recorder. The flow-through spectrophotometer used to detect peroxidase activity was a micro flow-through cell fitted in the center of the light path of a Sequoia Turner Model 340 Spectrophotometer and connected to a Linear Model 1201 strip-chart recorder.

Samples were delivered to the electrode or spectrophotometer by the flow injection system, using a 4-way flow injection valve from Rheodyne, Incorporated. The reactor was also a Rheodyne 4-way Rotary Teflon valve, with the sample loop removed and replaced by silicone rubber filled with solid-phase particles. Connections were made by using short sections of tight-fitting silicone tubing to sleeve the tubes to be joined.

A Perkin-Elmer Lambda Array 3840 photodiode array UV/VIS spectrophotometer operated by a Model 7300 Professional computer was used to record the UV/VIS spectra of enzymes, theophylline, insulin, and conjugates.

Reagents

The following materials were purchased from Sigma Chemicals Co. (St. Louis, MO): adenosine deaminase (ADA) (E.C. 3.5.4.4) type VI, horseradish peroxidase (HRP) (E.C. 1.11.1.7) type IV, anti-sheep whole serum (fractionated-rabbit; 42.5 mg protein/ml), anti-guinea pig whole serum (fractionated-rabbit; 30 mg protein/ml), N,N'-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), theophylline, bovine insulin, bovine serum albumin (BSA) (fraction V) and Sephadex G-200. Sheep anti-theophylline antiserum was obtained from Research Plus Inc. (Bayonne, NJ). Anti-insulin (guinea pig) was a product of Miles Lab., Inc. (Naperville, IL). Therapeutic Drug Control Sera were obtained from Fisher Scientific Co. (Detroit, MI), CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals (Uppsala, Sweden), Nylon 6/6 balls (3.2 mm) from Precision Plastic Ball Co. (Chicago, IL) and Carbonyldiimidazole(CDI)-activated glycerol-coated controlled pore glass (CPG) beads (250 Å pore, 120/200 mesh) from Pierce Chemicals Co. (Rockford, IL) were examined as solid-phase supports for preparation of the 2nd-antibody reactor. 2,2'-Azino-di-[3-ethyl-benzothiazoline-sulphonate(6)] (ABTS) was obtained from Boehringer Mannheim Co. (Indianapolis, IN). All other chemicals and solvents used were of analytical or reagent grade. Deionized water was used to prepare all solutions and buffers.

Buffers

The theophylline assay buffer was 0.05 M tris(hydroxyamino)methane-hydrochloric acid (Tris-HCl), pH 7.5. The assay buffer for insulin was 0.05 M NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.0. The

dilution buffers for theophylline and insulin conjugates, antibodies, and standards were prepared using the assay buffers containing 0.1 M NaCl, 0.10% (w/v) gelatin, 2.0% (w/v) bovine serum albumin, and 0.01% (w/v) sodium azide. The reactor regeneration buffer was 0.2 M glycine-HCl, pH 2.2. Bound ADA activity was measured by flowing a 0.25 mM adenosine solution, prepared in the theophylline assay buffer, through the reactor. HRP activity measurements were made using a solution containing 2.0 mM ABTS and 6.0 mM H_2O_2 prepared in 0.1 M sodium acetate/0.05 M NaH_2PO_4, pH 4.0.

Preparation of 2nd-Antibody-CPG Beads

The immobilization procedure was performed by suspending 400 mg of CDI-activated glycerol-coated CPG beads in a mixture of 1200 μl of 0.1 M sodium borate buffer, pH 8.5, and 300 μl of rabbit anti-sheep whole serum antiserum (antibody fraction) or sheep anti-guinea pig whole serum antiserum (antibody fraction) [10, 11]. This suspension was first degassed with ultrasonic vibration for 20 min, and then shaken for 2 days at 4°C. The remaining active hydroxyl groups on the beads were deactivated by treatment with 2 M ethanolamine. The suspension was then centrifuged and the beads were washed repeatedly with assay buffer. Based on absorption measurements at 280 nm, approximately 17 mg of total protein from each fractionated anti-sera was coupled to 1 g of CPG beads.

Preparation of 2nd-Antibody Reactor

The 2nd-antibody-CPG beads (approximately 120 mg, dry weight) were slurried in an assay buffer and packed into a silicone tubing (2.54 mm i.d. × 2.7 cm). A thin plug of glass wool was placed at each end of the reactor to prevent leakage and movement of the CPG beads by the carrier stream. The 2nd-antibody reactor was stored at 4°C between experiments and filled with assay buffer containing 0.01% (w/v) sodium azide.

Preparation of Theophylline-ADA Conjugates

Theophylline-8-butyric acid lactam was synthesized by the modified method of Cook [12]. To prepare the theophylline-ADA conjugates, four different amounts of the theophylline-8-butyric acid lactam were added to four solutions, containing 72 units of enzyme (previously dialyzed versus sodium bicarbonate buffer, 0.1 M, pH 8.5). After mixing overnight, the reaction mixtures containing the enzyme conjugates were dialyzed five times at 4°C against 0.05 M Tris-HCl, pH 7.5. Each conjugate was characterized by the degree of conjugation (moles of theophylline/mole of enzyme) and by its residual enzymatic activity. The degree of conjugation was estimated from calculations involving absorbance measurements at two absorption maxima of the conjugate (230 nm and 283 nm).

Preparation of Insulin-HRP Conjugate

The alpha and epsilon amine functional groups of insulin were modified by treatment with succinic anhydride to form carboxylic acid derivatives [13].

The resulting derivatized insulin was then reacted with NHS to form N-hydroxysuccinimide esters [14]. The enzyme conjugation reaction was performed by adding the required amount of NHS-activated insulin to 4 mg of HRP in 800 μl of 0.1 M sodium bicarbonate buffer, pH 8.5, at 4°C with stirring overnight. The reaction mixture was then dialyzed against 0.05 M phosphate buffer, pH 7.0, and further purified (to remove unreacted insulin) by gel chromatography (Sephadex G-200, 40 × 1 cm) in the same buffer. Each 1.5 ml of effluent was assayed for HRP and protein content by monitoring absorbances at 403 nm and 280 nm. The first protein peak (highest MW) was the desired insulin-HRP conjugate. This conjugate fraction was diluted (1 : 4) and then used in the subsequent FIIA procedures without further purification.

Theophylline and Insulin 1st-Antibody Dilution Curves

In order to determine the binding capacity of the 1st-antibodies for the conjugates, varying concentrations of 1st-antibody were incubated with a fixed amount (50 μl) of the appropriately diluted conjugate. The final volume of each vial was brought to 100 μl with assay buffer containing BSA. The vials were incubated for 7 min, and this mixture was introduced into the 2nd-antibody reactor (anti-sheep serum or anti-guinea pig serum). The bound enzymatic activity was measured by flowing the appropriate substrate solution through the reactor.

Preparation of Standard and Sample Solutions

Working theophylline standards were prepared by diluting a stock solution of theophylline ($10^{-3} M$ in H_2O) with Tris-HCl buffer containing BSA and EDTA. These solutions were freshly prepared for each standard curve. Fisher Diagnostics Therapeutic Drug Controls (TDC) were used as control sera. The high and low levels of TDC were diluted 1 : 400 with Tris-BSA buffer containing 1 mM EDTA before assaying for theophylline.

The insulin stock solution was 10 mg of bovine insulin (Sigma, lot 57F-0242, 25.6 I.U./mg) dissolved in 1 ml of 0.03 N HCl which was then diluted to 100 ml with phosphate-BSA buffer. This stock solution was stored frozen. The working standards were diluted from this stock solution daily with the phosphate-BSA buffer.

Theophylline and Insulin Dose-Response Curves

The typical FIIA procedure used for both analytes was as follows: A known amount of enzyme-labeled analyte (35 μl) and a given amount of standard free analyte (35 μl) were mixed with 35 μl of 1st-antibody and 35 μl assay buffer. This mixture was shaken for 7 min. The mixture was then loaded into the sample loop (valve 2 in Fig. 2) and injected into the 2nd-antibody-CPG reactor. The actual exposure time for this mixture with the 2nd-antibody column was very short (approximately 5 s); after which the carrier buffer stream washed off the unbound species for 4 min. When the valve of

the reactor injector was opened, substrate solution passed through the reactor. The products of the enzymatic reactions were measured by down-stream with an ammonium ion-selective electrode (theophylline) or spectro-photometer (insulin). Results were recorded on a strip-chart recorder. The system was regenerated by washing with 0.2 M glycine-HCl buffer, pH 2.2, for 2 min to dissociate the complex between the 1st antibody and immo-bilized 2nd-antibody, after which the system was ready for another sample.

Dose-response curves were prepared by plotting detected ammonium ion concentration versus logarithm of the theophylline concentration in the standards. The insulin dose-response curve was prepared by plotting absorbance at 405 nm versus logarithm of the bovine insulin concentration in the standards.

Results and Discussion

Initially, several solid-phase support materials were evaluated for prepa-ration of the 2nd-antibody reactor. CNBr-activated Sepharose 4B is a common support used in affinity chromatography; however, because of poor mechanical strength, Sepharose 4B could not be employed here due to the relatively high flow rates used in the FIIA system [15]. Smaller and more durable Nylon balls and CPG beads were also examined. Unfortunately, severe non-specific protein adsorption on the normal CPG beads [16], and the lower surface area of the Nylon balls, limited their use in the immu-noassay system. Ultimately, 1,1-carbonyldiimmidazole(CDI)-activated, glycerol-coated CPG beads were chosen as the solid-phase material to prepare the 2nd-antibody reactor. This material consists of normal CPG beads first treated with a hydrophilic, glycophase layer which removes the ionic and denaturing properties of the uncoated glass. The glycophase layer is then activated with CDI to produce a support which has high capacity for covalently binding proteins, low non-specific protein adsorption, and excellent long-term stability [17]. Other advantages include its high mechanical strength (enabling high flow rates) and greater surface area (compared to non-porous supports). Once reacted with the appropriate 2nd-antibody reagent, the glycerol-coated CPG beads could be stored at 4°C in phosphate buffer, pH 7.0, with 0.01% (w/v) NaN$_3$ for several months with little or no change in 1st-antibody binding ability.

The analytical utility of any EIA method is controlled, to a large extent, by the quality of the enzyme-analyte conjugates used as competitive binding reagents. Therefore, we expended considerable effort, particularly in the case of the theophylline assay, to prepare conjugates with substantial residual catalytic activity. For example, Table 1 summarizes the character-istics of four theophylline-ADA conjugates synthesized with varying initial ratios of theophylline-8-butyric acid lactam and ADA. As shown, as the number of theophylline molecules attached to the ADA increases, the activity of the conjugate decreases. For most of the studies reported here, a 1 : 200 dilution of conj-1 was used. In the case of the insulin method, final size exclusion purification of the insulin-HRP conjugate yielded a very broad band of protein. The protein fraction with the highest molecular

Table 1. Characteristics of theophylline-adenosine deaminase conjugates

Conjugate	Initial ratio of[a] theophylline/enzyme	Degree of conjugation	% Residual[b, c] activity
Conj-1	25	1.4	94.2
Conj-2	50	2.0	86.5
Conj-3	125	3.2	78.8
Conj-4	250	3.9	77.9

[a] Refers to molar ratios of theophylline lactam to adenosine deaminase in the conjugation reaction mixture

[b] Measured by optical absorbance change at 260 nm [18], not by electrode methods

[c] Measured relative to the same concentration of ADA used for conjugation reaction but without addition of theophylline lactam (i.e. blank)

weight, indicating high degree of insulin attachment to HRP, was used in the assays (diluted 1 : 4).

A typical timing sequence and signal output of the proposed FIIA assay scheme is depicted in Fig. 3 (for the theophylline assay). After equilibrating the 1st-antibody, conjugate, and sample or standard for a brief yet carefully
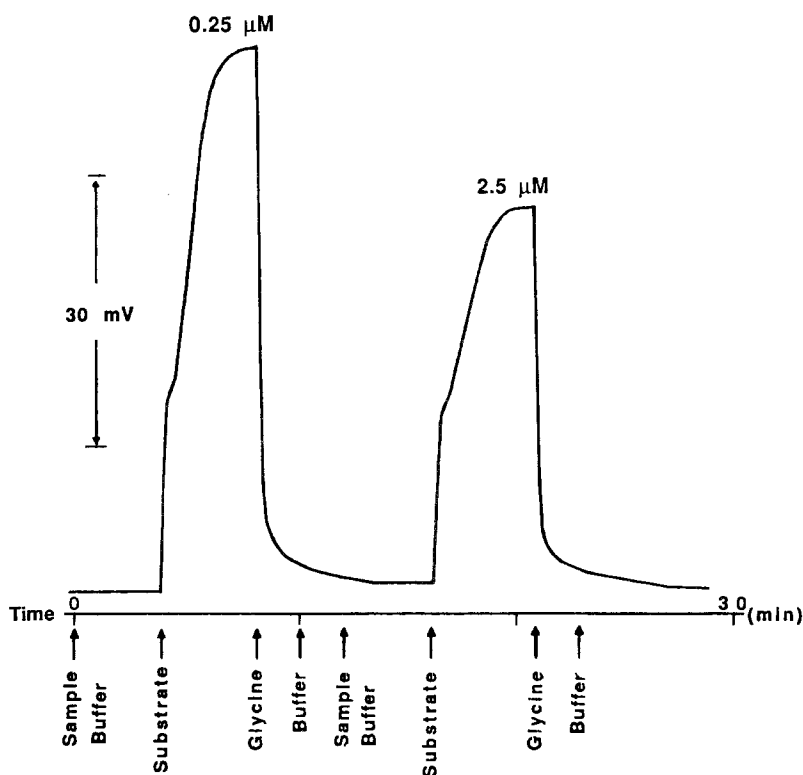

Fig. 3. Typical strip-chart recording showing the valve cycling and resulting output for the theophylline enzyme-linked FIIA system. Levels of theophylline refer to concentrations within injected reaction mixture

controlled period, the 100 µl of the reaction mixture is introduced into the sample loop (valve 2 in Fig. 2) and injected through the 2nd-antibody reactor with assay buffer as the carrier stream solution. A third rotary valve controls whether the assay buffer or regeneration buffer (glycine) passes through the reactor. Substrate solution is diverted through the reactor by turning the valve which houses the reactor. The resulting peak heights are directly (insulin) or logarithmically (theophylline) proportional to the enzymatic activity bound to the reactor's solid-phase. Although manually operated for these experiments, improvements in assay precision and sample throughput could be achieved if valve operations were computer controlled. The shoulders on the rising portion of the substrate-response curves shown in Fig. 3 are due to trace levels of ammonium chloride added to the Tris-HCl wash buffer in order to speed the response time of the ammonium ion-selective electrode toward low levels of NH_4^+ produced from the ADA reaction.

Dose-response curves for theophylline were prepared by plotting the percent of bound enzymatic activity (relative to zero-dose analyte, B/B_0) vs. logarithm of analyte. For insulin, direct measurement of product absorption (405 nm) was used as an indicator of bound enzyme activity. Figs. 4 and 5 illustrate typical curves obtained. The working range for theophylline was (0.025 µM—0.25 µM) and 1—250 µg/ml for insulin (concentrations in reaction mixture injected into system). For the most part, detection limits in these FIIAs are controlled by the amount of soluble 1st-antibody required to bind an amount of enzyme conjugate that yields a significant detector
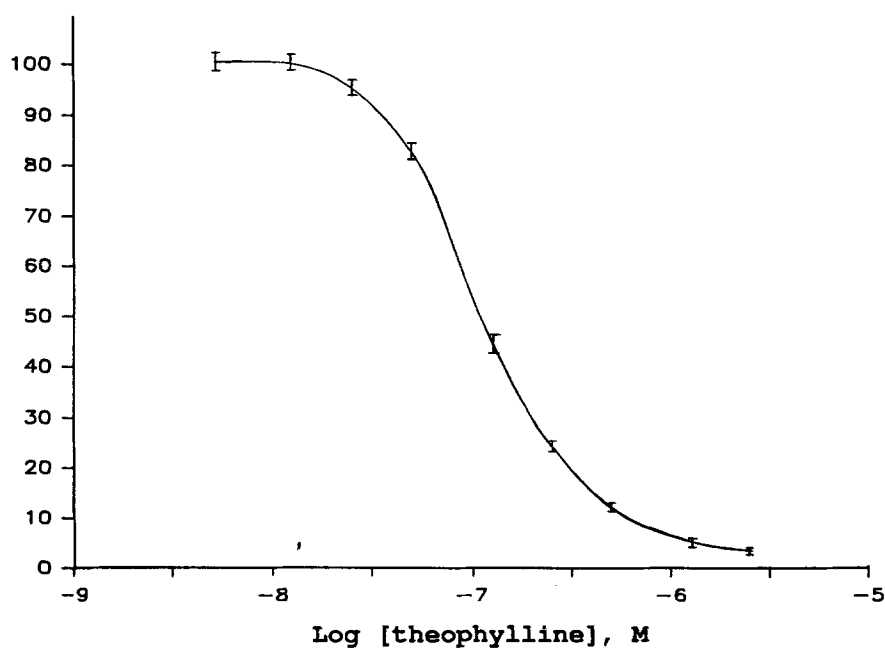

Fig. 4. Representative dose-response curve for theophylline FIIA system. Ranges shown are for duplicate measurements of each standard. Levels of theophylline refer to concentrations within injected reaction mixture
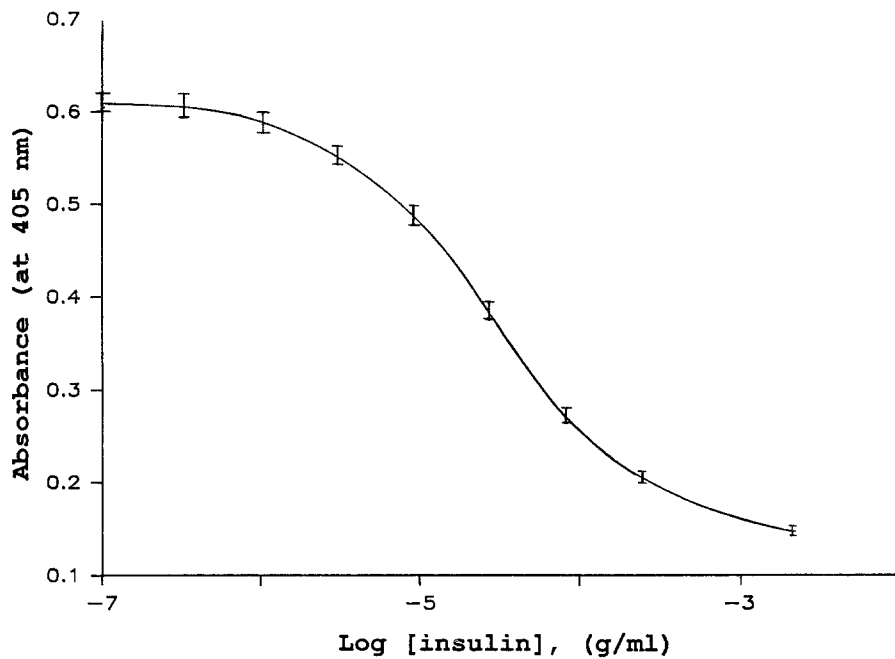
Fig. 5. Typical dose-response curve for detection of insulin using immobilized 2nd-antibody-based FIIA system. Ranges shown are for duplicate measurements of each standard
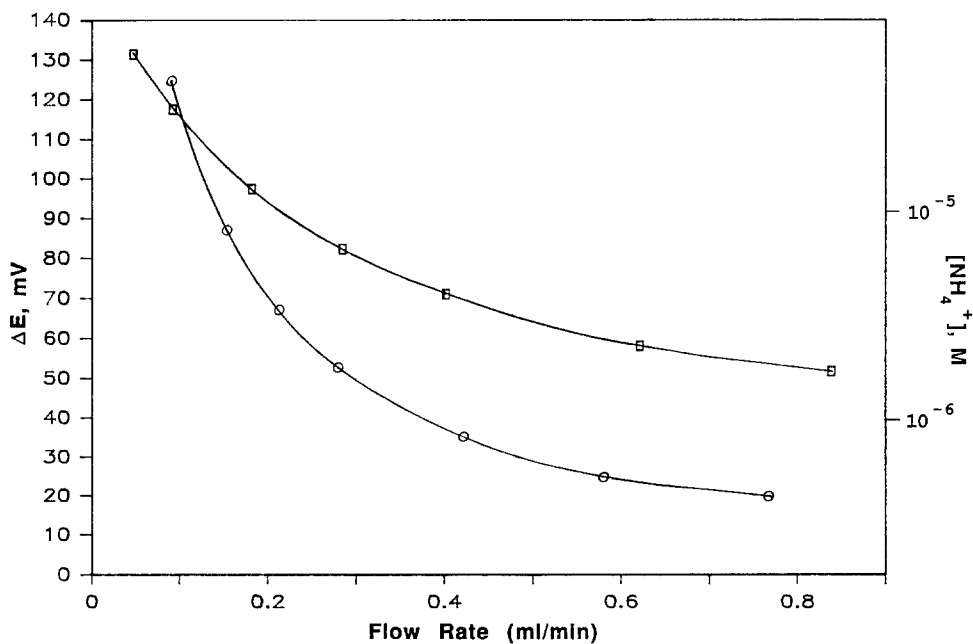

Fig. 6. Effect of carrier buffer (□) and substrate (○) flow rates on zero-dose peak signals observed for theophylline FIIA system

signal (for zero-dose analyte) upon flowing substrate through the reactor. This zero-dose signal is, in turn, related to the kinetics of the enzymatic reaction in the reactor and the binding of the 1st-antibody-enzyme conjugate complex to the immobilized 2nd-antibody. As shown in Fig. 6, for a given amount of the antibody and conjugate (theophylline system), the zero-dose signal decreases as the flow rate of either the substrate stream or sample injection stream (buffer) is increased. This is due to the non-equilibrium nature of the reaction processes within the reactor. At higher flow rates of sample and substrates (desired to achieve greater sample throughput), to increase the zero-dose signal, higher concentrations of 1st-antibody and enzyme-ligand conjugate must be used. Increasing these reagents tends to diminish the detection capabilities of the system. Improved detection limits concomitant with the desired faster flow rates can only be achieved if a more sensitive detection method is used to detect bound enzyme activity (e.g., fluorescence).

Aside from optimizing the flow-rates to obtain a proper balance in terms of detection limits and sample throughput, the composition of the flowing solutions, particularly the substrate, is also important. For example, in the case of detecting the activity of the bound insulin-HRP conjugate, the concentrations of the flowing substrates (H_2O_2 and ABTS) and the pH of this solution must be optimized to generate the maximum absorption signal (at 405 nm) from the bound conjugate. Moreover, the substrate concentrations must remain stable during the course of a given calibration. For this reason, the HRP substrate solution reservoir was kept at $0°C$ in the dark while the FIIA system was operating. The length of pump tubing between this solution and the reactor was made sufficiently long so that ambient temperature equilibration could take place before the solution passed through the reactor. While 6 mM H_2O_2 and 2 mM ABTS proved optimal in terms of substrate concentrations, the pH optimum for the HRP reaction (with ABTS as substrate) is only pH 4.0 [19]. Initially, we assumed that such a low pH would completely dissociate the bound conjugate and, with time, help degrade the binding ability of the 2nd-antibody reactor. However, we ultimately found that these concerns were not realized due to the relatively short time (2—3 min) that the substrate actually flows through the reactor.

The method and speed of regenerating the 2nd-antibody reactor was also studied. Whereas the pH 4.0 buffer used to prepare the HRP substrate solution did not rapidly dissociate the bound enzyme conjugates and/or 1st-antibodies, further studies suggested that a 0.2 M glycine-HCl buffer, pH 2.2, was quite efficient for such purposes. The kinetics of this immunodesorption step is illustrated in Fig. 7 for the dissociation of the theophylline-ADA conjugate-1st-antibody complex. As can be seen, the signal generated from the adenosine substrate solution goes to zero after only 90 s of washing the reactor with the low pH buffer. Such treatment, repeated for every sample or standard, is not detrimental to the performance of the FIIA system. Indeed, 2nd-antibody reactors were run for over 1 month without any significant decrease in the amount of enzyme-conjugates bound to the solid-phase at zero-dose of analyte. Such results do not imply that the absolute binding ability of the 2nd-antibodies is maintained
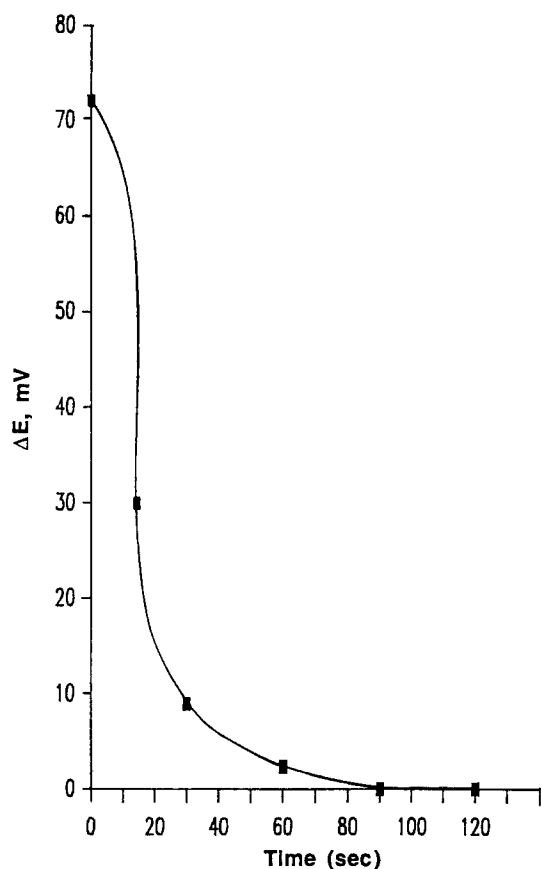
Fig. 7. Effect of contact time between glycine-HCl buffer and 2nd-antibody reactor on dissociating bound theophylline-ADA conjugate

after repeated treatment with pH 2.2 buffer. However, since the amount of 2nd-antibody sites is in great excess to the amount of limiting 1st-antibody binding sites used in the assays, substantial loss in binding ability can be tolerated without observing a change in the magnitude of the analytical

Table 2. Cross-reactivities of theophylline FIIA system

Compound	% Cross-reactivity[a]	
	FIIA	RIA[b, c]
Theophylline	100	100
Caffeine	5.0	4.2
Theobromine	0.3	0.09
Xanthine	<0.01	<0.01
Hypoxanthine	<0.01	<0.01
Uric acid	<0.01	<0.01

[a] Determined by dividing the concentration of theophylline at 50% of the response, by the concentration of analog that yields 50% of the maximum response
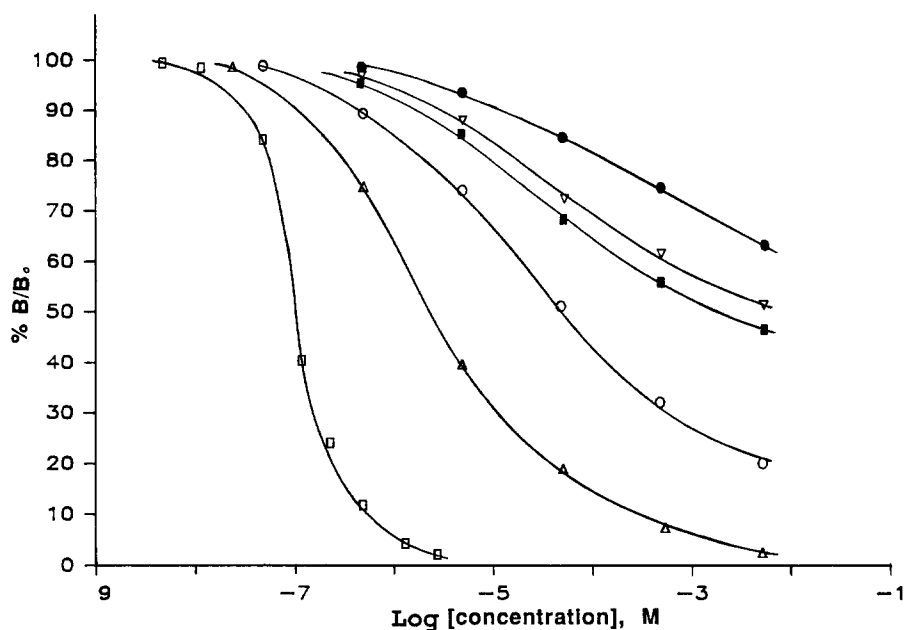
[b] From ref. [11]

[c] From ref. [19]

Fig. 8. Dose-response curves for xanthine analogs in theophylline FIIA system; (□) theophylline, (△) caffeine, (○) theobromine, (▽) xanthine, (●) hypoxanthine, (▮) uric acid

signals. We believe this is the primary advantage of using immobilized 2nd-antibodies to perform the separation step in flow-through EIA methods.

To demonstrate the analytical application of the proposed FIIA approach, more detailed studies were performed with the theophylline system. Fig. 8 illustrates the dose-response of this method toward several xanthine analogs. The percent cross-reactivity for each is tabulated in Table 2. The observed selectivity pattern is dependent on the association constants of the 1st-anti-theophylline antibody towards the analogs relative to theophylline. The selectivity of this FIIA system agrees well with what has been observed previously using polyclonal anti-theophylline antibodies in an RIA theophylline method [20], and appears suitable for direct determinations of theophylline in serum. Indeed, Table 3 compares the results obtained using the FIIA-EIA system to values found by several commercial theophylline methods on two control sera. Recovery studies were also performed in which additional theophylline was spiked into the "low" serum control. As shown in Table 4, the amounts added could be recovered with good accuracy.

In summary, a new heterogeneous enzyme-linked FIIA system has been described. The unique feature of this system is the use of an immobilized 2nd-antibody reactor column to separate 1st-antibody bound enzyme-analyte conjugates. By careful control of the initial reagent equilibrium times, the non-equilibrium binding and enzymatic reactions which occur in the reactor can be utilized to achieve relatively rapid total assay times (compared to other heterogeneous systems). Via the use of more sensitive

Table 3. Comparison of results for determination of theophylline in low and high serum controls[a]

Method	Low (μg/ml)[b]	High (μg/ml)[b]
Abbott TDX	4.6 ± 1.5	28.6 ± 5.6
Ames TDA Manual	4.0 ± 0.8	29.0 ± 4.3
Dade STRATUS	4.7 ± 1.6	30.1 ± 6.1
DuPont ACA	4.0 ± 1.5	26.3 ± 5.9
HPLC	4.7 ± 1.6	28.9 ± 4.3
IL MONACH	4.4 ± 1.0	29.0 ± 4.3
Pharmacia Diag. EIA	4.8 ± 1.3	32.5 ± 6.5
Roche COBAS	4.4 ± 1.6	26.6 ± 6.8
Syva EMIT	4.4 ± 1.6	26.6 ± 6.7
Present FIIA System	4.8 ± 0.4^{c}	32.2 ± 2.2^{c}

[a] EIA values from Fisher Therapeutic Drug Control Sera data sheet
[b] ± 2 standard deviation
[c] Average of four determinations ± 2 standard deviation

Table 4. Recovery of standard theophylline additions to a drug control serum

Added (μg/ml)	Found (μg/ml)[a]	% Recovery
0	4.8	—
10.0	14.6	98.6
20.0	24.2	97.5

[a] Average of three determinations

detection methods and fully automated reagent dispensing and valve controls, we believe that the proposed FIIA method could become an attractive approach for performing heterogeneous EIAs on a routine basis.

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