A competitive enzyme-linked immunoabsorbent assay based on the flow-injection amperometric detection of p-aminophenol has been investigated with use of the materials and general procedure of a commercial kit for the determination of theophylline in human serum. The antibody is immobilized on glass beads, and the enzyme label is alkaline phosphatase (EC 3.1.3.1). The high currents generated during the electrochemical detection allowed a rapid (35 min) and simple determination of theophylline throughout its therapeutic range (10–20 mg/L) and also in the subtherapeutic range (detection limit of about 80 µg/L).

**Additional Keyphrases:** alkaline phosphatase • electrochemical immunoassay • p-aminophenyl phosphate

Enzymes, when used as labels in immunoassay methods, allow the development of diverse assay protocols, with adaptability to both homogeneous and heterogeneous assays. Moreover, they provide a signal amplification that is especially useful at low analyte concentrations. The technique used to detect the enzyme product is a critical factor in the development of an enzyme immunoassay method. Most routine clinical methods are based on the formation of colored or fluorescent products, which are detected spectrophotometrically.

The application of electrochemical detection in enzyme immunoassays is under active investigation (1–8), given the potential for combining low detection limits with good selectivity. Both potentiometric and amperometric methods have been developed for use with homogeneous and heterogeneous assays (6). The coupling of immunoassay with liquid chromatography with electrochemical detection, or with flow-injection analysis with electrochemical detection (FIA-EC), has allowed the development of extremely sensitive assays (9, 10).

Although homogeneous procedures are faster and simpler than heterogeneous assays, the wide variety of compounds present in biological samples limits the use of electrochemical detection for this assay format. Because a high potential is required for the detection of enzyme-generated NADH, certain electroactive substances in biological samples, e.g., ascorbic acid, uric acid, and acetaminophen, can contribute to a high background signal. Proteins can also foul the electrodes by adsorption. Electrode protection (by use of a precolumn) to prevent fouling, the isolation of analytical signal (via a chromatographic column), and the use of low electrode potential have been investigated as remedies for these problems (7, 8). On the other hand, use of heterogeneous assays, which require an additional separation step to dispose of all the sample material except that bound to the antibody, would also avoid electrochemical interferences from the extraneous sample components.

A recently developed commercial heterogeneous enzyme immunoassay method is the EZ-BEAD™ kit (Immunotech Corp., Boston, MA 02134) for determining the bronchialator drug theophylline in serum. This heterogeneous solid-phase competitive-binding enzyme immunoassay is based on competition between theophylline in the patient's sample and a theophylline–alkaline phosphatase (EC 3.1.3.1) conjugate in the reagent for a limited number of binding sites on a bead coated with a highly specific anti-theophylline antibody. The amount of theophylline in the patient's serum is inversely proportional to the amount of conjugate bound to the bead, which is determined by the amount of phenol enzymatically generated from phenyl phosphate. As marketed, the phenol produced in the method is detected spectrophotometrically by the colored complex formed when potassium ferricyanide (stopping reagent) is added.

Here we assess the feasibility of using FIA-EC in the EZ-BEAD procedure for a theophylline assay by using p-aminophenyl phosphate (PAPP) as the enzyme substrate to generate p-aminophenol. The latter compound is detected by its oxidation current at a glassy carbon electrode in the FIA-EC system. The electrode reaction monitored is as follows:

\[ \text{H}_2\text{N}-\text{HN}==\text{O} + 2\text{H}^+ + 2e^- \rightarrow \text{HN}-(\text{fe}) + \text{OH} \]

**p-aminophenol**  **p-quinone imine**

**Materials and Methods**

**Reagents and Instrumentation**

EZ-BEAD enzyme immunoassay kits for theophylline were purchased from Immunotech Corp. 2-(Ethylamino)ethanol (EAE) was obtained from Aldrich Chemical Co. (Milwaukee, WI 53201). Because concentrated EAE is a toxic material (ORL-MAM LD50: 1200 mg/kg), caution should be taken to ensure safe handling and disposal (11). The PAPP was synthesized from p-nitrophenyl phosphate (Boehringer Mannheim Biochemicals, Indianapolis, IN 46250), by catalytic hydrogenation in a hydrogen shaker (Parr Instruments Co., Inc., Moline, IL), with use of palladium on activated carbon (Aldrich) as a catalyst (12). The p-aminophenol was purchased from Sigma Chemical Co. (St. Louis, MO 63178); abnormal control serum was from Ciba Corning Diagnostics Corp. (Irvine, CA 92714).

Glass disposable test tubes (12 × 74 mm) were a product of Fisher Scientific (Pittsburgh, PA 15219). Electronic digital pipets (from Rainin Instrument Co., Inc., Woburn,
MA 01801) were used to transfer the solutions. Electrochemical analyses were performed with flow-amperometric equipment from Bioanalytical Systems (BAS, West Lafayette, IN 47906). The amperometer was a BAS Model LC-4A. The thin-layer electrochemical cell had a glassy carbon working electrode, an Ag/AgCl (3 mol/L KCl) reference electrode, and a stainless-steel auxiliary electrode. The injection volume was 20 μL, and the tubing from the injection to the detector was 20 cm long.

To prepare 1 mol/L EAE solution, used as the mobile phase, we added 100 mL of the concentrated EAE to 900 mL of doubly-distilled water and adjusted to pH 9.8. We used a flow rate of 1.0 mL/min. PAPP solution (5 mmol/L) was prepared in 1 mol/L EAE solution. p-Aminophenol was detected at +0.1 V. All the potentials are referred to an Ag/AgCl reference electrode.

Procedure

We modified the EZ-BEAD kit procedure, pipetting 25 μL of the kit's standards or patients' serum samples and 200 μL of the kit's alkaline phosphatase–theophylline conjugate sequentially into a series of 12 × 75 mm test tubes. An antibody-coated bead was added to each tube, then incubated for 30 min at room temperature. Each bead was washed three times with water to remove any unbound material and placed in a test tube containing 1 mL of PAPP solution instead of the kit's phenyl phosphate solution. After the recommended incubation time of 15 min, we injected 20 μL of this solution into the flow-injection system and recorded the oxidation current. If necessary (because of limitations in the upper current rate of the amperometer), we diluted the incubation solution with 1 mol/L EAE solution just before injection.

Serum samples from patients receiving theophylline therapy were analyzed by this method and also by a fluorescence polarization immunoassay procedure, Abbott TDx (13), used at the Toxicology Laboratory of University Hospital, Cincinnati.

Results and Discussion

Electrochemical Detection of p-Aminophenol

Alkaline phosphatase is a commonly used enzyme in electrochemical enzyme immunoassay, particularly with phenyl phosphate as substrate. The generated phenol is measured by its oxidation current at +0.80 V (4, 6). However, there are essentially two problems with the electrochemical detection of phenol: the high oxidation potential, which results in high background noise, and fouling of the electrode surface, owing to the electropolymerization of phenolic radicals at concentrations exceeding about 10⁻⁴ mol/L.

We have previously (8) described the use of PAPP as a better substrate than phenyl phosphate, because its product, p-aminophenol, is more easily oxidizable (at about +0.1 V) than is phenol and does not foul the electrode, even at high concentration.

However, an important problem encountered with p-aminophenol is its susceptibility to air oxidation, which is faster at the high pH (9.0–10.0) essential for optimum activity of alkaline phosphatase. We therefore assessed the decay rate of the p-aminophenol signal in different buffers and pHs (Figure 1). In ammonium carbonate (Figure 1d), as reported previously (8), p-aminophenol signal decays so quickly at pH 9.8 (optimum for alkaline phosphatase activity) that the assay must be performed at pH 9.0 (Figure 1c), with use of short incubation periods. Adding oxalic acid, which reportedly prevents air oxidation of ascorbic acid (14), to the ammonium carbonate solution substantially slows the decay rate of p-aminophenol (Figure 1b). However, as seen in Figure 1a, EAE (1 mol/L, pH 9.8) is an even better buffer to minimize the air oxidation of p-aminophenol. In fact, although not shown, there is no significant decay of the p-aminophenol signal in this medium for at least an hour at room temperature.

At the same time, EAE, because of its phosphate-acceptor ability, reportedly is one of the best buffers for enhancing activity of alkaline phosphatase (15). Using the analysis of Cornish-Bowden and Eisenthal (16), we calculated K_m and V_max for the theophylline–alkaline phosphatase conjugate in EAE and ammonium carbonate at pH 9.0. K_m and V_max were 17.39 mmol/L and 50.63 nA/min, respectively, in EAE, and 1.03 mmol/L and 6.97 nA/min, respectively, in ammonium carbonate. The use of EAE allows a faster reaction rate, although K_m is increased in this buffer. We chose a PAPP concentration of 5 mmol/L for the immunoassay procedure.

The rate of nonenzymatic hydrolysis of PAPP is slow, only about 0.29 mmol/L per minute. Fresh substrate solutions were prepared daily.

Figure 2 shows hydrodynamic voltammograms of p-aminophenol in EAE, and of EAE only, at the glassy carbon electrode in the flow amperometric system. For p-aminophenol, the region at which the current reaches a plateau is at +0.1 V; moreover, because EAE becomes electroactive above +0.2 V, the working potential chosen was +0.1 V.

A standard calibration plot of p-aminophenol as mea-
sured with the flow-injection system shows a very wide linear response range for peak current vs p-aminophenol concentration, from $5 \times 10^{-6}$ to $10^{-4}$ mol/L ($y = -0.58 nA + 49.56 nA \cdot \mu mol^{-1} L \cdot x, r = 0.999, S_{x,y} = 0.15 nA \cdot \mu mol^{-1} L, n = 15$). The detection limit was about $10^{-8}$ mol/L, defined as the p-aminophenol concentration for which the signal is twice the blank signal. Ten repeated injections of a p-aminophenol standard of $10^{-6}$ mol/L gave reproducible peaks with a coefficient of variation (CV) of 0.8%.

**Electrochemical Enzyme Immunoassay**

The enzymatically generated p-aminophenol was easily monitored by electrochemical oxidation, giving very large currents after 15-min incubation. The responses over 20 min for three theophylline concentrations were linear (Figure 3). As expected, the presence of more theophylline results in a smaller slope, because less conjugate is bound to the bead.

A calibration plot was then constructed according to the manufacturer's instructions (Figure 4). The y-axis values are equal to 100 C/C0, where C and C0 are the currents for the standard and 0.0 mg/L standard, respectively. Each point is the average of two separate determinations. The points lie on the straight line, $y = 100.4 - 44.73x$ ($r = 0.999, n = 5$).

The reproducibility of the results, i.e., interassay precision, is shown in Table 1 for each standard used.

**Comparison of Methods: Patients' Samples**

Serum samples from patients receiving theophylline therapy at University Hospital were analyzed by the FIA-EC method and the Abbott TDx, but not on the same days. A good correlation was obtained within and below the therapeutic range, with a linear-regression coefficient ($r$) of 0.98 (Figure 5). Results for the single sample that was analyzed in the toxic range (>20 mg/L) also gave a good correlation, differing by 3.5% between procedures.

**Shortened Assay**

The high concentrations of p-aminophenol generated during 15 min of substrate incubation meant that a shorter procedure was possible, involving only 5 min of substrate incubation. A calibration plot of p-aminophenol oxidation current vs theophylline concentration is shown in Figure 6, in the typical nonlinear form of competitive immunoassays. The currents shown range from 150 to 720 nA, high enough to suggest that the incubation time could be shortened yet further if an automated system was used to maintain precision.

**Detection Limit**

The detection limit of the manufacturer's prescribed procedure was claimed to be about 1.1 mg/L. Electrochemical detection, in the modified procedure, had the same detection limit, well below the therapeutic concentration range (10–20 mg/L). The detection limit in the shortened electrochemical assay was about 1.2 mg/L. The high sensitivity of electrochemical detection also permitted us to dilute both the theophylline standards (with abnormal
control serum) and the theophylline–alkaline phosphatase solution (with Tris-saline buffer, 0.1 mol/L, pH 10.4). Two calibration plots were constructed and compared for 10- and 100-fold dilutions of both reagents. A lower detection limit (about 80 μg/L) was obtained with the 100-fold dilution procedure. This demonstrates that assays could be performed not only at lower cost (about half the cost per test vs the manufacturer's assay), but also with a substantially smaller sample volume (0.25 μL) than is usually used for this assay (25 μL).

In conclusion, electrochemical detection based on the oxidation current of p-aminophenol applied to the EZ-BEAD enzyme immunoassay procedure for theophylline analysis in serum is feasible and practical. Because this is a heterogeneous procedure, there is no interference by electroactive substances, or electrode fouling; therefore, electrode protection is not required. The high sensitivity of the electrochemical detection provides a faster procedure than does the manufacturer's and also allows for determinations of the analyte in the μg/L range.

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