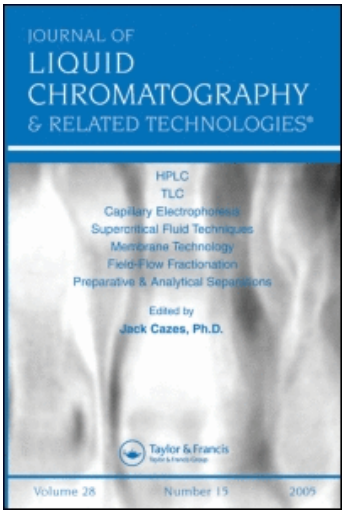

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LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION OF PHENOL AND NADH FOR ENZYME IMMUNOASSAY

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ABSTRACT

Enzyme immunoassays based on chromatographic separation and amperometric detection of an enzyme generated product have been investigated. These assays combine the selectivity of the antigen/antibody reaction with the high sensitivity of thin layer amperometry. The feasibility of utilizing LCEC as a detection scheme was demonstrated using the Syva EMIT® kit for phenytoin. NADH production by glucose-6-phosphate dehydrogenase was monitored following a homogeneous procedure. Heterogeneous assays were developed for alkaline phosphatase labeled species which were based upon LCEC determination of phenol. Assays were designed for a common serum glycoprotein (orosomucoid) and a clinically important drug (digoxin). Detection limits approach the pg/mL level and as such may prove fruitful in the quantitation of numerous antigens of clinical interest.

INTRODUCTION

Immunoassay is a widely used analytical technique, especially for the analysis of biological samples in clinical laboratories (1,2). One form of immunoassay is based on the competition between an antigen (the species to be determined) and labeled

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antigen for a limited amount of highly specific antibody-binding sites. The predominant immunoassay technique is radioimmunoassay in which the label is a radioactive isotope that is detected by various disintegration counting techniques.

Numerous other labels have been explored for use in immunoassay, and a few of these are now used routinely (3,4). For example, an enzyme label that catalyzes the production of nicotinamide adenine dinucleotide (NADH), which is detected spectrophotometrically, is widely used (5,6). The low detection limit of fluorescence has also stimulated the development of immunoassays based on fluorescent labels (7,8).

We and other research groups have investigated the potential of electrochemical techniques for analytical detection in immunoassay (9-13). One strategy that we have pursued involves the use of an enzyme label for catalyzing the production of an electroactive species. Liquid chromatography with electrochemical detection (LCEC) has proved to be an effective analytical technique for use with such enzyme immunoassays. We describe here the application of LCEC to the detection of phenol and NADH which are catalytically generated by enzyme-labeled antigens.

MATERIALS AND METHODS

Heterogeneous Enzyme Immunoassay

Polystyrene cuvettes were purchased from Gilford Instruments, Cleveland, OH 44135. Alkaline phosphatase Type VII, orosomucoid (OMD), antibody to OMD and digoxin were obtained from Sigma Chemical Co., St. Louis, MO 67138. Orosomucoid was coupled to alkaline phosphatase by a known procedure (14). The digoxin alkaline phosphatase conjugate was obtained from Immunotech Corp., Cambridge, MA 02139. Phenyl phosphate was purchased from Calbiochem-Behring Corp., La Jolla, CA 92037. Digoxin antisera was a gift from the Center for Disease Control, Atlanta, GA.

The buffers used in the heterogeneous assay were phosphate buffered saline with Tween 20 (PBS-Tween): 4.08 g KH_2PO_4, 8.37 g K_2HPO_4, 500 µL Tween 20, dilute to 1.0 L and adjust the pH to 7.4 with 5.5 M NaOH; 0.05 M sodium carbonate: 2.93 g $NaHCO_3$, 1.59 g Na_2CO_3, dilute to 1.0 L (pH=9.6); and 0.1 M potassium phosphate: 6.16 g KH_2PO_4, 9.59 g $KHPO_4$, dilute to 1.0 L (pH=7.0). Antibody coating solutions of 10 µg/mL for the digoxin assay and 1.0 µg/mL for the OMD assay were made by dissolving the appropriate amount of antibody in 0.05 M carbonate buffer containing 0.02% sodium azide. Antigen-enzyme conjugate dilutions of 1/125 and 1/100 were used for the digoxin and OMD alkaline phosphatase conjugates respectively. PBS-Tween was used to dilute the enzyme conjugates. Standard solutions of digoxin (50 pg/mL - 5.0 ng/mL) and OMD (1.0 ng/mL - 200 ng/mL) were prepared in human serum and PBS-Tween, respectively. The enzyme substrate solution employed in this study was made by dissolving 0.20 g phenyl phosphate and 0.30 g $MgCl_2 \cdot 6H_2O$ in 100 mL of carbonate buffer.

Electrochemical analyses were performed with flow-amperometric equipment from Bioanalytical Systems Inc. (BAS), W. Lafayette, IN 47906. The amperometer was a BAS Model LC 3. The electrochemical cell was a 2.0 mil thin-layer cell with a carbon paste working electrode, Ag/AgCl reference electrode and either a glassy carbon or stainless steel auxiliary electrode. All electrochemical analyses were performed at +870 mV using 0.1 M phosphate buffer (with 0% to 4.0% MeOH) as the eluent and a flow rate of 1.2 mL/min. A 12 cm by 4 mm Knauer column (Unimetrics, Anaheim, CA 92801) was dry packed with LC-4 37-44 µm pellicular C-18 packing material and placed between the pump and injection valve to presaturate the mobile phase. A 5 cm by 2 mm precolumn was slurry packed with 10 µm irregularly shaped RSiL C-18 material (Alltech Associates, Deerfield, IL 60004) and was used for the separation of phenol from the assay buffer. A Milton Roy pump (Model 29-290) and a 20-µL injection loop were used.

For the heterogeneous assay cuvettes were coated with specific antibody through passive adsorption by incubation with antibody coating solutions for 12 hrs. The coating solution was then aspirated and the cuvettes were washed 3X with PBS-Tween, allowing the PBS-Tween to stand in the cuvettes for 10 min during each wash. The antigen solution (375 µL for digoxin, 400 µL for OMD) and the antigen-enzyme conjugate dilution solution (25 µL for the digoxin conjugate, 100 µL for the OMD conjugate) were added to the cuvettes and incubated for 6 hrs (digoxin) and 12 hrs (OMD). The contents of the cuvettes were then aspirated and the cuvettes washed consecutively with 1X PBS-Tween, 1X PBS-Tween (5 min), and 2X carbonate buffer. The enzyme substrate solution was then added to each cuvette and incubated for 25 min and 60 min for digoxin and OMD, respectively. For the digoxin assay the enzyme reaction was stopped by the addition of 25 µL of 5.5 M NaOH to each cuvette. Directly before injection 25 µL of 5.5 M HCl was added to each solution. For the OMD assay the enzyme reaction was stopped by simply removing the substrate solution from the cuvette with a pipet.

Homogeneous Immunoassay

EMIT® phenytoin assay kits were purchased from Syva Co., Palo Alto, CA 94304. NADH (N8129) was a product of Sigma Chemical Company. Phosphate buffer (0.1 M, pH=6.5) was made from K_2HPO_4 and KH_2PO_4. Syva Model 1500 pipetter-diluter was used for sample handling procedures.

Electrochemical analyses were conducted using BAS flow-amperometric equipment. Conditions were similar to those reported in the previous section except that the working electrode was glassy carbon (+750 mV vs Ag/AgCl) and the analytical column was a 25 cm X 4.6 mm Knauer column dry packed with LC-4 (37-44 µm) ODS phase packing material. Periodically, the working electrode was polished with alumina, then conditioned and pretreated before use

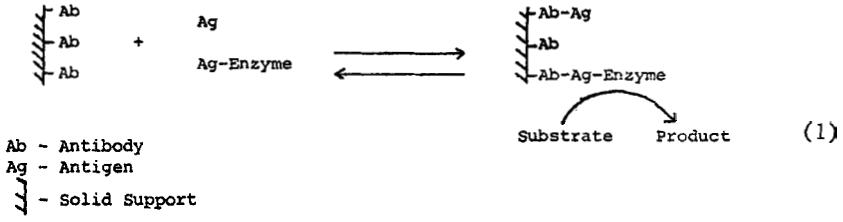
by cycling the electrode between +1.5 V and -1.5 V at a scan rate of 100 mV/s for 20 min. A potential of +1.5 V was applied for 2 min, followed by 2 min at -1.5 V. This was repeated twice. Each day the background current was allowed to decay to a constant value before starting analyses.

For the homogeneous assay the EMIT® assay components were diluted according to the manufacturer's instructions except that before injection the reaction mixture was further diluted 121-fold with phosphate buffer at 2, 5, 8, or 11 min after mixing. This was carried out to prevent fouling of the electrode surface by adsorbed NAD^+. **This dilution step also effectively stops the** enzymatic generation of NADH. The diluted solutions (20 μL) were injected into the LC at 3, 6, 9, and 12 min, respectively.

RESULTS AND DISCUSSION

Heterogeneous Enzyme Immunoassay

Heterogeneous enzyme immunoassays are used routinely for the clinical determination of small quantities (ng/mL-pg/mL) of biologically important compounds in serum and urine. In a competitive assay format an enzyme labeled antigen and an unlabeled antigen compete for a limited number of antibody binding sites, which are attached to a solid support (Equation 1). **After the**

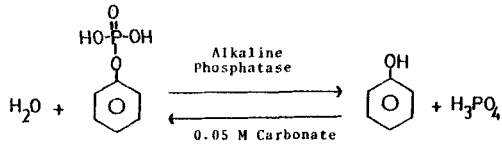


(1)

reaction in Eqn. 1 has reached equilibrium, the labeled and unlabeled antigen bound to the solid phase antibody are separated from the material free in solution. The amount of bound

labeled antigen is then determined by allowing the enzyme to react with its substrate for a given length of time. Enzyme generated product is then detected by an appropriate analytical method. In the competitive format the amount of bound labeled antigen will be inversely related to the amount of unlabeled antigen present. A standard curve is constructed using known concentrations of antigen, and the concentration of antigen in an unknown sample is found by reference to this standard curve.

Competitive heterogeneous immunoassay methodology has been developed utilizing alkaline phosphatase (AP) as the labeling enzyme. AP catalyzes the conversion of an electroinactive substrate (phenylphosphate) to an electroactive product (phenol), as shown in Equation 2.



(2)

A cyclic voltammogram for phenol at a carbon paste electrode (CP-OE) is shown in Figure 1. The irreversible 1-electron oxida-

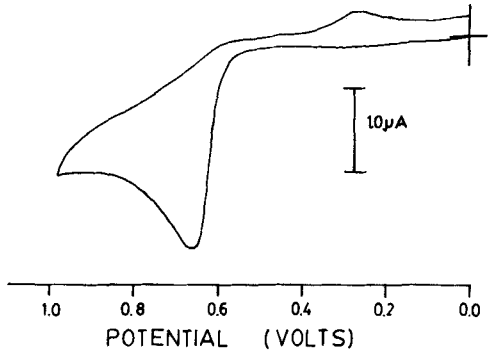


FIGURE 1. Cyclic voltammogram of 1.5×10^{-4} M phenol in 0.05 M carbonate buffer (pH = 9.6); carbon paste working electrode, Ag/AgCl reference electrode, scan rate = 10 mV/s.

tion wave of phenol was observed at +670 mV vs Ag/AgCl in a 0.1 M carbonate supporting electrolyte. Repetitive scans indicated that electropolymerization of the phenolic radical resulted in fouling of the electrode surface.

LCEC methodology was utilized to avoid fouling and increase sensitivity. A CP-OE working electrode was employed in a thin layer configuration. A 5 cm by 2 mm precolumn packed with 10 μm C-18 was used to retain phenol from the assay buffer (Figure 2). The hydrodynamic voltammogram for the determination of phenol is depicted in Figure 3. Maximum current response was obtained at potentials greater than +850 mV, and a potential of +870 mV was chosen for subsequent studies. The LCEC detection of phenol has a linear dynamic range from 9.0×10^{-9} M to 9.6×10^{-6} M (slope = 0.57 nA/nmol, y-intercept = -0.30 nA, r = 0.999).

The immunoassay reaction was carried out, and the bound material was separated from "free" by simply aspirating the

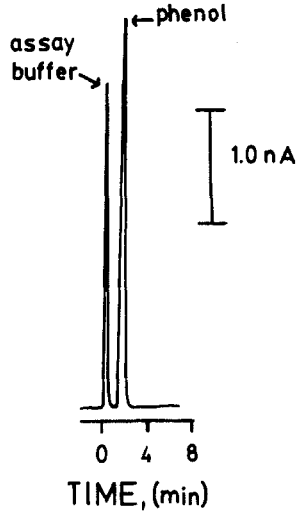


FIGURE 2. LCEC chromatogram of a 20 μL injection of 5.3×10^{-7} M phenol in 0.05 M carbonate buffer. Flow rate; 1.2 mL/min; eluent, 0.1 M phosphate buffer (pH = 7.0).

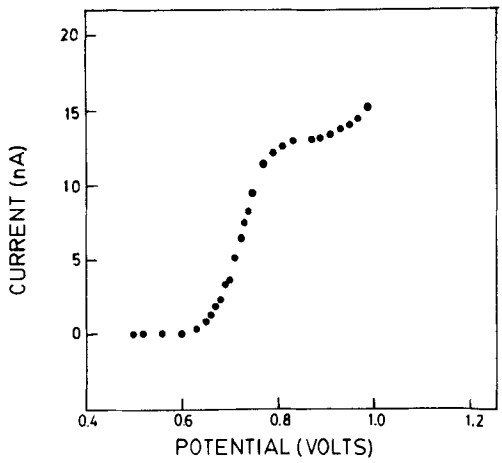


FIGURE 3. Hydrodynamic voltammogram for a $7.0X10^{-7}$ M phenol solution. See conditions for Figure 2.

assay solution. The amount of enzyme labeled antigen bound to the solid phase antibody was then determined by adding enzyme substrate to the cuvette and detecting the phenol generated after a given time interval.

The effect of varying concentrations of digoxin and OMD on the amount of their enzyme labeled analogues bound by the solid phase antibody are shown in Figure 4 and Figure 5. As the concentration of digoxin or OMD was increased the amount of the corresponding enzyme labeled material bound by the solid phase antibody decreased via the equilibrium reaction of Eqn. 1. The decrease in the amount of bound labeled antigen resulted in a decline in the amount of enzymatically generated phenol. The quantitative detection of phenol, following the heterogeneous LCEC procedure, provides the basis for the analytical determination of digoxin and OMD.

Homogeneous Enzyme Immunoassay

Enzyme immunoassays with electrochemical detection are being developed with glucose-6-phosphate dehydrogenase as the enzyme

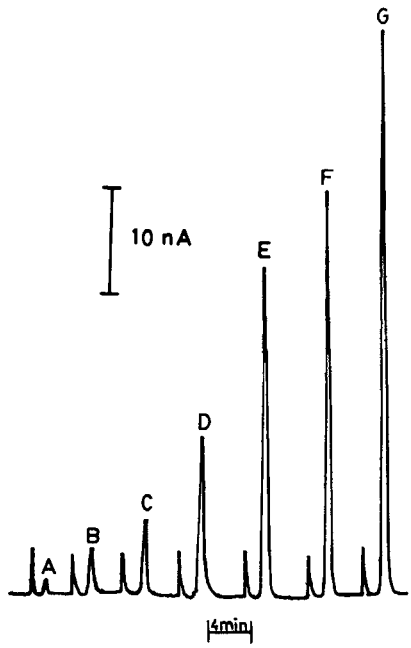
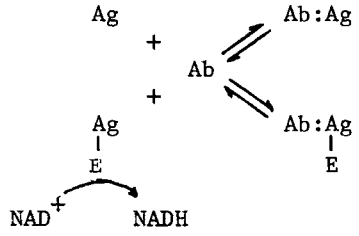


FIGURE 4. LCEC analysis of a series of digoxin standard solutions in serum. Concentration of digoxin in serum: A = 2.0 ng/mL, B = 1.0 ng/mL, C = 0.5 ng/mL, D = 0.25 ng/mL, E = 0.10 ng/mL, F = 0.05 ng/mL and G = 0.0 ng/mL.

label. These assays depend on the competition of an antigen (Ag) and an enzyme labeled antigen (Ag-E) for a limited amount of specific antibody (Ab) as shown in Equation 3. The free Ag-E catalyzes



(3)

the reduction of NAD^+ to NADH, whereas the catalytic activity of the bound (Ab-Ag-E) label is substantially diminished. Consequent-

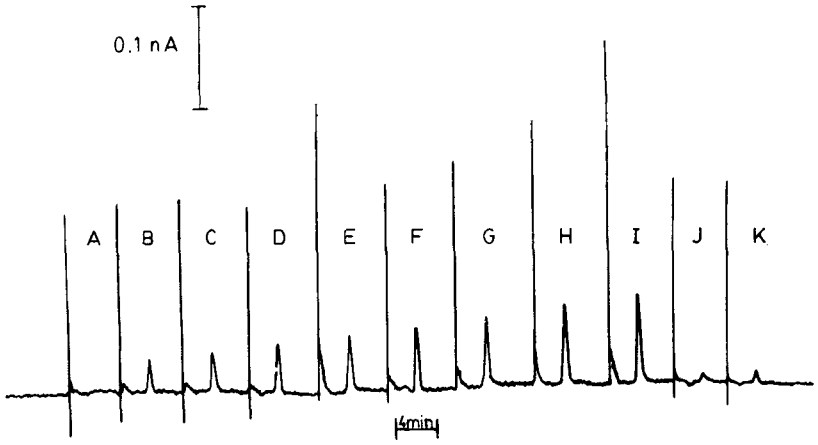


FIGURE 5. LCEC analysis of a series of OMD standard solutions: A) 0.05 M carbonate buffer, B) 200 ng/mL, C) 100 ng/mL, D) 60 ng/mL, E) 10 ng/mL, F) 5 ng/mL, G) 2.5 ng/mL, H) 1.0 ng/mL and I) 0.75 ng/mL. The addition of AP alone (J) or OMD-AP in cuvettes coated with non-specific IgG (K) showed little activity and hence minimal non-specific adsorption.

ly, a greater concentration of Ag in the sample or standard results in a greater concentration of free Ag-E, following competitive equilibration with a limited amount of Ab. This in turn results in more rapid production of NADH. There is a commercially available kit for drug analysis, EMIT® (15), based on the spectrophotometric determination of the rate of NADH production.

An alternative method of detecting NADH is by electrochemical oxidation. Figure 6 shows a cyclic voltammogram of NADH at a glassy carbon electrode. An oxidation wave with a peak potential of +0.85 V was observed. Reduction of the electrogenerated NAD^+ occurred at -1.2 V, making the system electrochemically irreversible. Curve A in Figure 7 shows a hydrodynamic voltammogram for NADH oxidation obtained by flow injection analysis (FIA). It is apparent from this curve that optimum sensitivity for FIA is obtained by maintaining the potential more positive than +0.8 V.

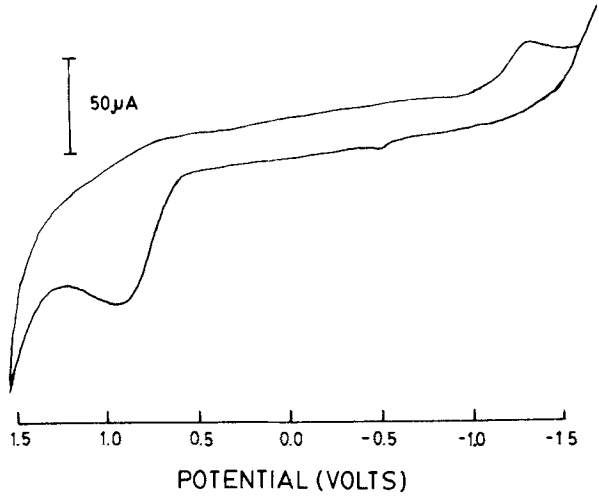


FIGURE 6. Cyclic voltammogram of $8.2X10^{-3}$ M NADH in 0.1 M phosphate buffer (pH = 6.5); glassy carbon working electrode, Ag/AgCl reference electrode, scan rate = 10 mV/s.

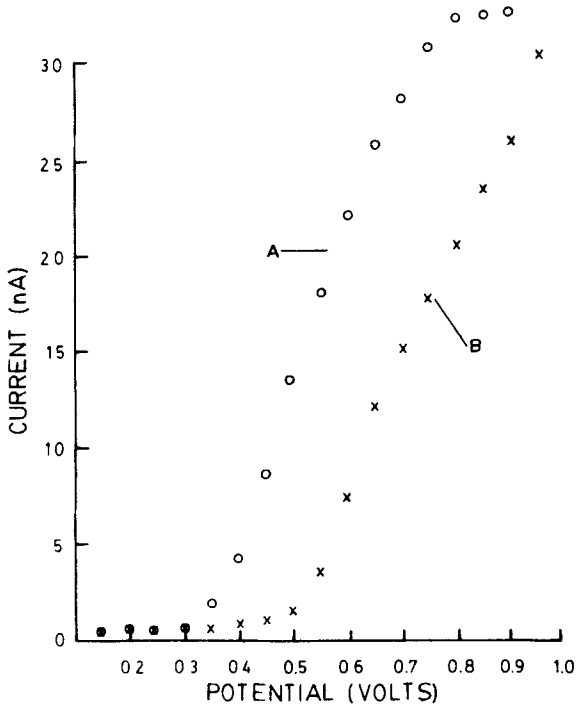


FIGURE 7. Hydrodynamic voltammograms for A) NADH and B) antibody by flow injection analysis.

Reproducible peak heights were obtained with repeated injections of NADH so long as the concentration of NADH was maintained at 10^{-6} M or below. Repetitive injections of higher concentrations resulted in continuously diminishing peak heights due to electrode fouling by adsorbed product(s) of NADH oxidation. Thus, adjusting conditions so that NADH concentrations are maintained below 10^{-5} M was essential to the successful amperometric detection of NADH at glassy carbon.

A standard curve for the FIA detection of NADH showed a linear response for peak current vs. concentration of NADH over the range 9.0×10^{-7} M to 1.8×10^{-6} M (slope = 0.63 μA/μmol, y-intercept = 0.28 nA, r = 0.999). The detection limit for this system was 1×10^{-7} M. Eleven repeated injections of a 8.7×10^{-6} M standard gave reproducible peak currents with a CV of 1.5%.

Although NADH can be determined with simple flow injection analysis, the use of a reversed-phase C-18 column was found to be necessary to implement the immunoassay scheme for several reasons. First, slight retention of NADH to separate it from the current response accompanying the void volume enabled lower detection limits to be achieved. Second, Ab was electroactive at the optimum potential for NADH determination. Curve B in Figure 7 depicts a hydrodynamic voltammogram for Ab. Third, Ab and protein components in serum samples were found to gradually foul the electrode surface by adsorption, resulting in diminished peak currents for NADH. This behavior is illustrated in Figure 8. Repetitive injections of NADH were performed first followed by repetitive injections of Ab. A systematic decrease in the Ab peak was observed and is attributed to electrode fouling by adsorbed Ab. Subsequent injection of the same amount of NADH as originally injected gave the substantially diminished peak currents shown in Figure 8. The insertion of a C-18 column overcame these difficulties by retarding the NADH peak about 30 s from the void volume peak (1.5 min). The LC column also irreversibly retained

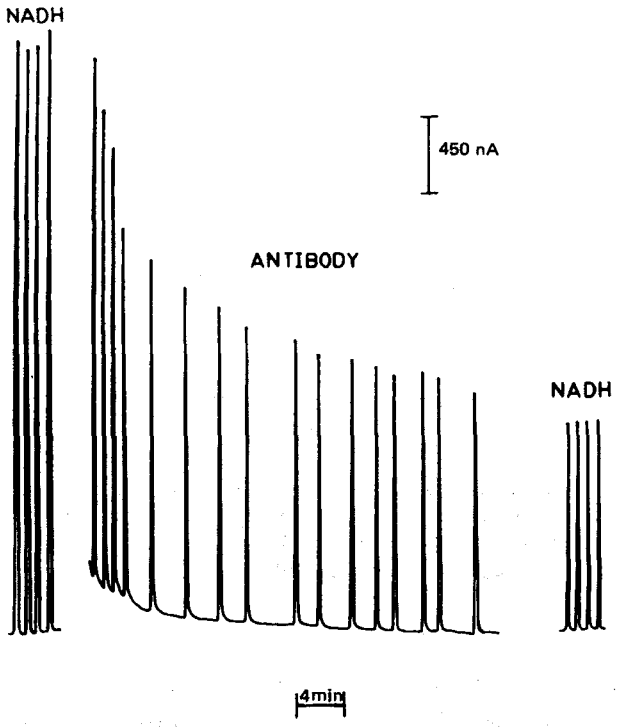


FIGURE 8. Flow injection analysis performed sequentially on aliquots of a NADH solution, an antibody solution and then the same NADH solution.

Ab and prevented electrode-fouling by serum proteins. In general, a C-18 column would last for about 80 - 100 injections during immunoassays. A protein-saturated column was immediately detectable by a decrease in the peak current for the enzymatic reaction. Columns could be regenerated by flushing with methanol or ethanol.

LCEC using a C-18 column and a thin-layer cell containing a glassy carbon working electrode (maintained at 0.75 V) was found to be an effective means of determining NADH, and hence phenytoin, based on the EMIT® assay. Phenytoin standards are mixed with the various assay reagents according to the assay protocol. *The rate*

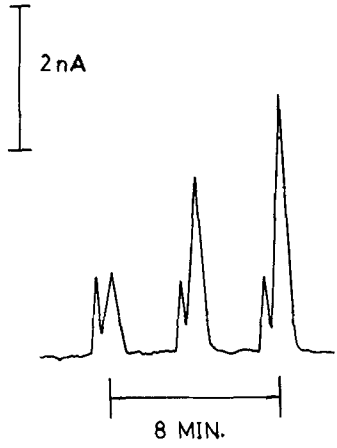


FIGURE 9. Current response for a 30 µg/mL phenytoin standard injected at 3, 6 and 9 min after mixing. The first smaller peak in each case is from the Tris-HCl buffer, and the second peak is NADH.

of NADH production for each standard is determined by injecting aliquots of the assay mixture at various times into the LCEC. Chromatograms showing the rate of NADH production with time for a 30 µg/mL phenytoin standard are shown in Figure 9. The rate of NADH production, for a given standard, can be evaluated by measuring the slope of a peak height (nA) vs time (min) plot. After computing the rates for each phenytoin standard, a calibration plot can be generated for unknown sample analysis (10).

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REFERENCES

1. Masbacher, C.J. Now and Future RIA, Res. Dev. July, 20 (1977).

2. Ebersole, R.C. and Chait, E.M. Clinical Analysis, Anal. Chem. 53, 682A (1981).

3. Nakamura, R.M. and Ditio, W.A. Nonradioisotopic Immunoassays for Therapeutic Drug Monitoring, Lab. Medicine, 11, 807 (1980).

4. Schall, R.F. and Tenoso, H.J. Alternatives to Radioimmunoassay: Labels and Methods, Clin. Chem., 27, 1157 (1981).

5. Voller, A.; Bartlett, A.; Bidwell, D.E. Enzyme Immunoassays with Special Reference to ELISA Techniques, J. Clin. Pathology, 31, 507 (1978).

6. Scharpe, S.L.; Cooreman, W.M.; Bloome, W.J.; Laekman, G.M. Quantitative Enzyme Immunoassay: Current Status, Clin. Chem., 22, 733 (1976).

7. O'Donnell, G.M. and Suffin, S.C. Fluorescence Immunoassays, Anal. Chem., 51, 33A (1979).

8. Lidofsky, S.D.; Imasaka, T.; Zare, R.N. Laser Fluorescence Immunoassay of Insulin, Anal. Chem., 51, 1602 (1979).

9. Wehmeyer, K.R.; Halsall, H.B.; Heineman, W.R. Electrochemical Investigation of Hapten-Antibody Interactions by Differential Pulse Polarography, Clin. Chem., 28, 1968 (1982).

10. Eggers, H.M.; Halsall, H.B.; Heineman, W.R. Enzyme Immunoassay with Flow-Amperometric Detection of NADH, Clin. Chem., 28, 1848 (1982).

11. Doyle, M.J.; Halsall, H.B.; Heineman, W.R. Heterogeneous Immunoassay for Serum Proteins by Differential Pulse Anodic Stripping Voltammetry, Anal. Chem., 54, 2318 (1982).

12. Weber, S.G. and Purdy, W.C. Homogeneous Voltammetric Immunoassay: A Preliminary Study, Anal. Lett., 12(B1), 1 (1979).

13. Yuan, C.; Kaun, S.S.; Guilbault, G.G. Immunoelectrochemical Assay for Creatine Kinase Isoenzyme MB, Anal. Chem., 53, 190 (1981).

14. Avrameas, S. Coupling of Enzymes to Proteins with Glutaraldehyde, Immunochem., 6, 43 (1969).

15. Bastiani, R.J. The EMIT System: A Commercially Successful Innovation, Antibiotics, Chemother., 26, 89 (1979).