

Heterogeneous Enzyme Immunoassay of Alpha-Fetoprotein in Maternal Serum by Flow-Injection Amperometric Detection of 4-Aminophenol

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A sandwich-type heterogeneous enzyme immunoassay with flow-injection analysis for alpha-fetoprotein (AFP) in human serum has been developed. 4-Aminophenol, the product of enzymatic reaction, is detected amperometrically. The interassay CV for this electrochemical enzyme immunoassay was <8.2%, with a minimum detection limit for AFP of 0.163 $\mu\text{g/L}$. The calibration curve had a linear range of 0.316–100 $\mu\text{g/L}$. Studies with 48 human maternal serum samples, comparing results by this method with those by a commercial kit, showed a good correlation ($r = 0.961$). This procedure provides an alternative method for determining low concentrations of AFP in human maternal serum.

Additional Keyphrases: *electrochemical techniques · fetal status · alkaline phosphatase*

Alpha-fetoprotein (AFP) is a developmental glycoprotein (67 kDa) normally produced in large quantities during embryonic development by the fetal liver and yolk sac and in small quantities by the fetal gastrointestinal tract (1, 2).² AFP enters the amniotic fluid through urination, passing through the placenta to enter the maternal circulation. At 12–14 weeks of gestation, amniotic fluid concentrations of AFP ordinarily peak at ~15–20 mg/L, after which they decline rapidly. Similarly, maternal serum AFP (MSAFP) concentrations ordinarily peak, at ~250 $\mu\text{g/L}$, between 32 and 34 weeks of gestation (3). AFP concentrations in infant and mother decrease rapidly after delivery, with a half-life of four to six days. At six to eight months postpartum, AFP concentrations in serum reach the adult value (<3 $\mu\text{g/L}$) (4, 5). However, AFP may reappear if its production is restimulated by unusual conditions, e.g., in association with certain types of carcinomas (4).

Increased concentrations of MSAFP have been observed in fetuses affected by an open neural-tube defect (NTD), either anencephaly or spina bifida (6–8). NTDs result from the failure of the developing neural tube to fuse, a process that ordinarily occurs by the fifth week of gestation. Lower than normal concentrations of MSAFP have been associated with Down syndrome (9), a condition characterized by mental retardation and physical abnormalities caused by trisomy of chromosome 21. Clinical interpretations of MSAFP concentrations in screening for NTD and Down syndrome in early pregnancy have been reported (9–14).

Several manufacturers offer AFP test kits, typically involving enzyme immunoassay or, less commonly, radioimmunoassay methodology (15, 16). Other techniques such

as fluorescence immunoassay, bioluminescence (17), and chemiluminescence (18) are being adapted for AFP analysis.

The current assay methods for amniotic fluid and MSAFP are used for assays of samples taken at about 15–21 weeks of gestation. This lower limit is defined by the low concentrations of MSAFP present before 14 weeks, the upper limit by the legal age at which abortion can be performed. Because of possible changes in this legal age, there may be a need to begin the screening process (i.e., MSAFP analysis) earlier in a pregnancy. In addition, the increasing use of chorionic villus sampling for detection of genetic abnormalities might make amniotic fluid from the early weeks of gestation more readily available for the analysis and early detection of NTDs than is currently the case. However, inaccurate assay results, such as are likely with present methods and the low AFP concentrations early in the gestation, not only impose unusual stress on the patient but also have a serious impact on the clinical decision. A much more sensitive assay than is currently available is therefore required.

For an enzyme immunoassay, the technique used to detect the enzymatic reaction product defines not only the detection limit, selectivity, sensitivity, and precision of the methodology but also the sample size, throughput, and the feasibility of automation. Most routine clinical methods are based on the formation of colored or fluorescent products, which are detected spectrophotometrically. The applications of flow-injection amperometric detection with different enzyme immunoassay formats have been reported (19–21). By being an interfacial rather than a bulk solution phenomenon, electrochemical detection is well-suited for scaling down for measurements on a small sample volume (e.g., microliters) with excellent detection limit, sensitivity, and precision. Besides, the flow-injection system can act as an "interface" to convert the existing batch enzyme immunoassays into automated forms. The combination of electrochemical detection and flow-injection analysis with enzyme immunoassay exhibits unique features of this approach (19–21).

In this study, we have developed an enzyme immunoassay with flow-injection amperometric detection for MSAFP in low concentrations, with a detection limit of 0.163 $\mu\text{g/L}$ and a linear dynamic range of three orders of magnitude.

Materials and Methods

Apparatus

The flow-injection amperometric detection system used for this work was a model 400 LCEC system [Bioanalytical Systems (BAS), West Lafayette, IN] without a separation column. This system consisted of an HPLC pump (BAS; PM-48), an amperometric detector (BAS; LC-4B), an amperometric flow cell (BAS; CC-4), and a temperature controller (BAS; LC-22A) (optional). The amperometric thin-layer cell had dual glassy carbon working electrodes, an Ag/AgCl (3 mol/L NaCl) reference electrode, and a stain-

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² Nonstandard abbreviations: AFP, alpha-fetoprotein; MSAFP, AFP in maternal serum; NTD, neural-tube defect; BAS, Bioanalytical Systems; and FIAEC, flow-injection analyzer-electrochemical detection system.

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less-steel auxiliary electrode (Figure 1). The sample loop was 20 μL and the flow rate was 1.0 mL/min. Data collection was by chart recorder. Although we report here the manual operation of the method, the flow-injection amperometric detection system can be easily automated by coupling to a BAS CMA/200 automated microsampler.

Sample dilution was performed by means of a sample dilutor from Fisher Scientific (Cincinnati, OH). A shaker-water bath (Model 127) was also from Fisher Scientific.

Materials

Mouse anti-human AFP IgG1 (cat. no. A-013-09, lot M-11939) and alkaline phosphatase (EC 3.1.3.1)-conjugated mouse anti-human AFP IgG1 (cat. no. Z-893-05, lot 14087-3A) were from Medix Biotech Inc. (Foster City, CA). AFP zero diluent and calibrators were from the Tandem[®]-E AFP antibody kit (cat. no. 4179, lot 990798; Hybritech Inc., San Diego, CA), as used in the comparison clinical assay. Human serum immunoassay control level 3 (lot 6000) was from Bio-Rad (Anaheim, CA). The Centers for Disease Control biological standard for AFP in mid-pregnancy maternal serum (cat. no. IS1080, lot 101780) was a gift from Dr. M. J. Adams, Jr. (Centers for Disease Control, Atlanta, GA). Maternal serum samples were obtained from the University Hospital of Cincinnati (Cincinnati, OH). Other materials were the same as in previous work (19).

Buffers and Solutions

Buffer I: sodium acetate/acetic acid, 100 $\mu\text{mol/L}$; Tween[®] 20, 0.5 mL/L; and sodium azide, 0.1 g/L, pH 4.0.

Buffer II: sodium acetate/acetic acid, 100 $\mu\text{mol/L}$; Tween 20, 0.5 mL/L; bovine serum albumin, 20 g/L; and sodium azide, 0.1 g/L, pH 4.5.

Buffer III: Tris, 0.1 mol/L; magnesium chloride, 1 mmol/L; and sodium azide, 0.1 g/L. The pH was adjusted to 9.0 with hydrochloric acid.

The primary coating solution of mouse anti-human AFP IgG used to create the capture surface was prepared from a 1.0 g/L stock solution by 400-fold dilution with buffer I. Mouse anti-human AFP-alkaline phosphatase conjugate solution was prepared from a stock solution (containing 0.2 g of AFP monoclonal antibody per liter) by 400-fold dilution with buffer II. 4-Aminophenyl phosphate (4 mmol/L), the enzyme substrate, was made up in buffer III immediately before use, to minimize nonenzymatic hydrolysis. The low-concentration AFP calibrator was prepared by dilution of high-concentration calibrator with the zero diluent from

the Tandem-E kit. Specimens having AFP >100 $\mu\text{g/L}$ were diluted with the zero diluent and re-assayed. The mobile phase for the flow-injection analysis was buffer III.

Method

In this heterogeneous sandwich enzyme immunoassay, we used a polystyrene Nunc-Immuno plate (Nunc, Roskilde, Denmark) as the solid phase. A 400- μL aliquot of the capture antibody coating solution was pipetted into each well and incubated for 10–14 h at room temperature to permit adsorption. The wells were incubated three times (2×5 min, 1×20 min) by pipetting 400 μL of buffer II into each well, then aspirating. We then added to each well 30 μL of AFP calibrators or specimens plus 300 μL of buffer II. The plate was placed in the shaking device and incubated for 2 h at room temperature. This was followed by three rinses with buffer II (400 μL) for 5 min each. After adding 300 μL of mouse anti-human AFP-alkaline phosphatase conjugate solution to each well, we incubated the samples for 3 h at room temperature in the shaker, then rinsed the wells three times with buffer III (400 μL , 5 min each) to remove unbound labeled antibody. The alkaline phosphatase activity remaining was determined by a 30-min reaction with 300 μL of the substrate solution. At the end of the reaction, we injected 20 μL of the reacted substrate solution into the flow-injection analyzer-electrochemical detection system (FIAEC). We measured the oxidation peak current of 4-aminophenol at an applied potential of +300 mV (vs Ag/AgCl) to construct the calibration curve.

Results and Discussion

Assay Conditions

In this immunoassay procedure, the capture antibody was adsorbed passively to the polystyrene solid phase. It was critical for the success of the assay to manipulate this adsorption so as to secure enough active capture antibody on the surface while minimizing the nonspecific adsorption of enzyme-labeled antibody by the same mechanism. Satisfying these criteria (results not shown) led to the use of buffers I and II as optimal in this assay. We used plateau incubation times for the assay; therefore, we consider it possible that assay times could be shortened.

Electrochemical Detection by Flow-Injection Analysis

The electrochemical characteristics of 4-aminophenyl phosphate and 4-aminophenol, the substrate and product, respectively, of the alkaline phosphatase used in the assay, have been discussed previously (19, 22). The 4-aminophenol generated by the hydrolysis of 4-aminophenyl phosphate can be oxidized, its limiting oxidation current being at potentials >+250 mV vs Ag/AgCl in FIAEC (buffer III), whereas 4-aminophenyl phosphate is electroinactive, even at +400 mV (22). The advantages of detecting 4-aminophenol are the low oxidation potential and the lack of fouling of the electrode surface, resulting in low background noise and excellent precision (19, 22). The electrochemical reaction monitored in the FIAEC system is as follows:

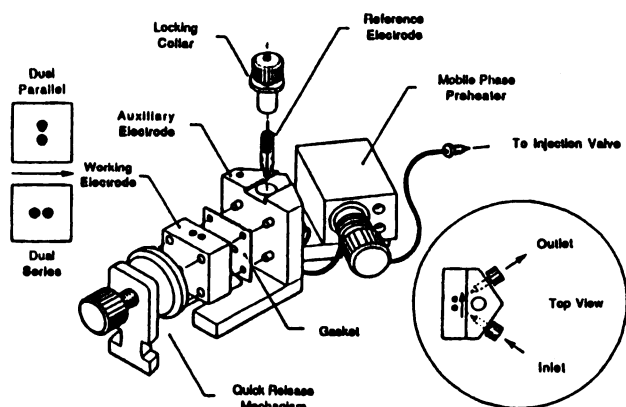
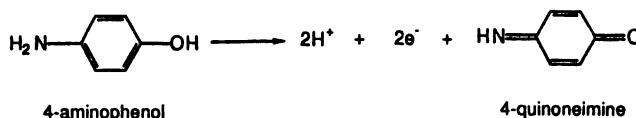


Fig. 1. Amperometric thin-layer cell for BAS electrochemical detectors (courtesy of BAS)

A consideration in using FIAEC is the presence of a nonfaradaic signal that coregisters with the analytical signal. The unwanted nonfaradaic signal exists principally because of the necessary difference in composition between the injected sample and the mobile phase, and results from the change in capacitance as this compositional difference passes over the electrode. The magnitude of the signal is a function of both the size of this compositional discrepancy and the oxidation potential at which the electrode is set. In this assay, to diminish the nonfaradaic current, we adjusted the composition of the mobile phase to minimize differences, and measured the 4-aminophenol at a low oxidation potential, resulting in a nonfaradaic signal of approximately only 8.7 nA. This is a critical feature of the assay because the low value of the nonfaradaic signal obviates the need for its chromatographic separation from the analytical signal, thereby permitting the use of FIAEC (19). Also, by improving the relative standard error of the zero-dose response in this way, we lowered the detection limit (23).

Analytical Performance

The electrochemical enzyme immunoassay exhibited good interassay precision over its linear range (CVs of <8.2%) (Table 1). The minimum detectable concentration of the assay, defined as the mean determination of the zero-dose AFP calibrator plus 2 SD, was 0.163 $\mu\text{g/L}$. The lowest measured (but not necessarily the lowest measurable) AFP calibrator was 0.32 $\mu\text{g/L}$, with a CV of 4.1%. In contrast, for the commercial AFP kits, the lowest minimum detectable concentration is reportedly 1.9 $\mu\text{g/L}$ (16). Note that the detection limit of the FIAEC assay here is limited not by the ability to detect the 4-aminophenol, for which the detection limit by FIAEC is 2.4×10^{-8} mol/L (19), but by the value of the zero-dose response (2.4×10^{-8} mol of 4-aminophenol per liter would be equivalent to 6.1×10^{-4} μg of AFP per liter in this assay).

The AFP standard calibration curve is shown in Figure 2. The linear range extended from the detection limit up to 100 μg of AFP per liter. Bio-Rad immunoassay control serum (human) at level 3, which contains over 64 constituents besides AFP, was measured after a twofold dilution with the zero diluent. We measured AFP at 159.6 $\mu\text{g/L}$, which fell within the acceptable range (115.0–175.0 $\mu\text{g/L}$) provided by Bio-Rad.

Comparison Assay

In a comparison study, we analyzed routine maternal serum samples from pregnant women for concentrations of AFP by both this assay and the Tandem-E AFP immunoassay (Hybritech), performed with the Photonera automated immunoassay analyzer in the Clinical Chemistry Laboratory, University Hospital of Cincinnati. The data from both assays for 48 samples (Figure 3) were

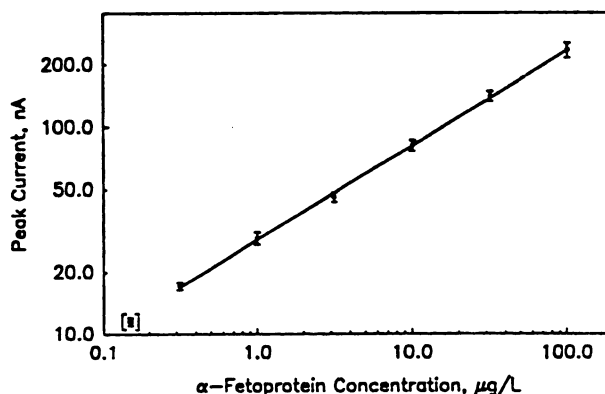


Fig. 2. FIAEC peak currents vs AFP calibrators in human serum [0] denotes the zero diluent ($n = 21$). Intercept = 1.46 $\log(\text{nA})$, slope = 0.456 $\log(\text{nA})/\log(\mu\text{g/L})$, $r = 1.00$. For points other than those from Table 1, $n = 4$; error bars denote SD

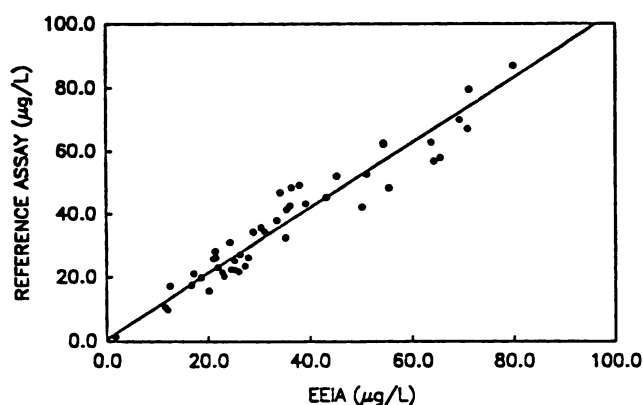


Fig. 3. Correlation between immunoassay of AFP (Tandem-E AFP, Hybritech) and electrochemical enzyme immunoassay of AFP in human maternal serum $n = 48$, intercept = 0.71, slope = 1.03, and correlation coefficient = 0.961. Points are included when calibrator CV was <10%

examined with Ligand Data Calc (v 2.12e), with use of the Method Comparison Statistics feature for immunochemical data reduction (David G. Rhoads Associates, Inc., Kennett Square, PA), which gave a correlation coefficient of 0.961 (Student's t -test $t = 2.50$, $P = 0.014$; i.e., there was only a 1.4% probability that the means of the two methods were statistically identical). This result is easily attributable to an inherent difference between the immuno-plates used in our assay and the immuno-beads of the Tandem-E AFP immunoassay. Note that the figures of merit for calibrators in serum assayed electrochemically (Figure 2 and Table 1) do not completely reflect the scatter of the correlation plot (Figure 3). Possible sources of scatter in AFP concentrations measured with the electrochemical enzyme immunoassay and with the comparison assay could result from sample storage, sample transportation, temperature fluctuation during immunological and enzymatic reaction, and the reproducibility of aspiration in each rinsing step. These uncertainties should be minimized by assaying the samples under exactly the same conditions and using automated equipment such as the Photonera automated immunoassay analyzer.

In conclusion, we have shown the feasibility of enzyme immunoassay for quantifying AFP in maternal serum with

Table 1. Interassay Precision of Electrochemical Enzyme Immunoassay of AFP

AFP calibrator, $\mu\text{g/L}$	n	Mean (SD) \bar{x}		CV, %
		$\mu\text{g/L}$	nA	
0.000	21	11.5 (0.5)		4.3
0.316	15	17.1 (0.7)		4.1
10.0	15	82 (5)		6.1
100	8	233 (19)		8.2

the use of flow-injection amperometric detection of 4-aminophenol. This method demonstrates a wide dynamic range, good interassay precision, and a lower minimum detection limit than the present commercial test kits for alpha-fetoprotein in maternal serum. It provides not only an alternative method for clinical use, but also another means for early detection of NTD or Down syndrome.

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