CLIN. CHEM. 33/11, 2000–2003 (1987)

Time-Resolved Immunofluorometric Assay of Alpha-Fetoprotein in Serum and Amniotic Fluid, with a Novel Detection System

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We describe a new "sandwich"-type nonisotopic immunoassay of alpha-fetoprotein (AFP) in serum and amniotic fluid. In the assay, AFP binds to a monoclonal antibody immobilized in a microtiter well and to a polyclonal soluble biotinylated antibody. A fluorescent product is created on the solid-phase after adding streptavidin labeled with a new Eu^{3+} chelate, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA), and excess Eu^{3+}. The fluorescent complex, monoclonal antibody–AFP–polyclonal antibody–biotin–streptavidin–BCPDA–Eu^{3+}, is quantified by nitrogen laser excitation at 337.1 nm, the emission at 615 nm being monitored in an especially designed gated fluorometer working in a time-resolved mode. This assay of AFP has a broad dynamic range (up to 1 mg/L), and is precise and accurate. The detection limit is ~0.1 μg/L. Results agree well with those obtained by established techniques.

Additional Keyphrases: *gated fluorometry · monoclonal antibodies · biotin–streptavidin binding · nonisotopic immunoassays · neural tube defects · mongolism*

Alpha-fetoprotein (AFP) is synthesized in the yolk sac during embryonic life and later in the parenchymal cells of the fetal liver.[4] A glycoprotein, its physical properties are similar to those of albumin. AFP concentration in amniotic fluid and fetal serum peaks at about 14 weeks of gestation and decreases thereafter, whereas AFP values continuously increase in maternal serum during pregnancy. Concentrations of AFP in both the maternal serum and amniotic fluid increase in the presence of an open neural tube defect (spina bifida, anencephaly), findings that form the basis of prenatal screening (*1–3*) usually performed with maternal serum at about 14 to 18 weeks of gestation. Because the predictive value of a positive test is low, it is usually necessary to confirm results by ultrasonic studies and by measuring AFP in amniotic fluid. Increased concentrations of AFP are also associated with hepatoma and nonseminomatous testicular tumors (*4*). Moreover, decreased concentrations of AFP in maternal serum have been associated with Down's syndrome with subsequent development of screening programs based on this connection (*5, 6*).

Assay of AFP in biological fluids and especially serum has traditionally involved radioimmunoassays (RIA) or immunoradiometric techniques (*7, 8*). Among alternative methodologies, luminescence (*9*), time-resolved fluorescence (*10*), and enzyme immunoassays (*11, 12*) are commercially available, for which the current detection limits are similar to or better than those of RIA or immunoradiometry.

We have developed a sensitive noncompetitive "sandwich"-type immunofluorometric assay (IFMA) for determining AFP in serum and amniotic fluid with use of a novel detection system. A monoclonal "capture" antibody is noncovalently immobilized in a microtiter strip (or plate) well. The detection antibody is a biotinylated affinity-purified antibody, and streptavidin labeled with a new europium chelate, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA, "Eurofluor S™"; Figure 1), is used as the fluorescent label. The resulting complex that forms, monoclonal antibody–AFP–polyclonal antibody–biotin–streptavidin–BCPDA–Eu^{3+}, is quantified on the dry surface of the well by excitation with a nitrogen laser beam; the specific delayed fluorescence is monitored at about 615 nm. The sensitivity, precision, and accuracy of the assay are comparable with or better than those of existing techniques.

Materials and Methods

Reagents. For wash solution we used a 9 g/L NaCl solution containing 0.5 mL of polyoxyethylene sorbitan monolaurate (Tween 20) per liter. The blocking solution, 0.1 mol/L sodium bicarbonate buffer (pH 8.3), contained 10 g of bovine serum albumin (RIA grade; Sigma Chemical Co., St. Louis, MO 63178), 20 g of sucrose, and 0.5 g of sodium azide per liter. To dilute the standards, we used 10 mmol/L phosphate buffer (pH 7.0), containing 10 mmol of EDTA, 50 g of the albumin, 0.1 g of sodium azide, and 0.1 g of thimerosal per liter. Stock 1 mmol/L $EuCl_3$ solution was prepared by dissolving the hexahydrate salt (Aldrich Chemical Co., Milwaukee, WI 53233) in HCl, 10 mmol/L.

AFP standards. Human AFP (InterMedico, Toronto, Canada) was calibrated against the international reference standard (72/227) for AFP. We prepared AFP standards in

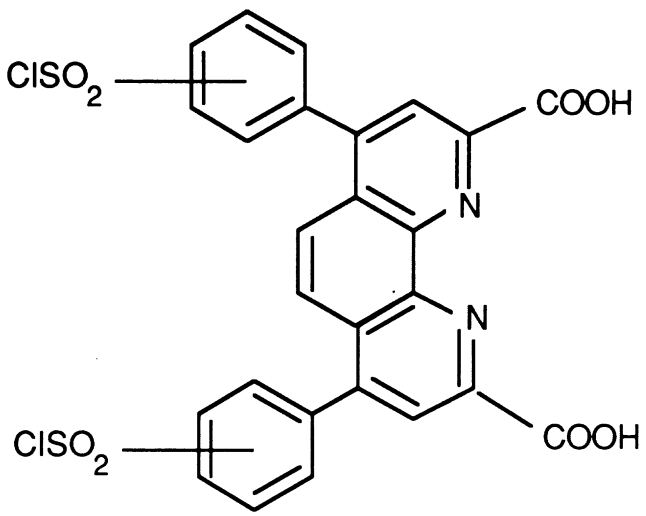

Fig. 1. Structure of the Eu^{3+} chelate 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid

The sulfonyl chloride groups bind covalently with available amino groups of streptavidin

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[4] Nonstandard abbreviations: AFP, alpha-fetoprotein; BCPDA, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid; IFMA, immunofluorometric assay.

Received June 2, 1987; accepted August 10, 1987.

concentrations ranging from 1 to 1000 μg/L in the standards-diluent solution.

Patients' samples and controls. Specimens of serum from pregnant women at various gestational ages, amniotic fluids, and serum from patients with liver- and testicular-tumors were obtained from the Toronto General Hospital. For controls we used human sera-based "Tri-level" ligand controls (Ortho Pharmaceuticals, Toronto, Canada). We diluted amniotic fluid 50-fold in the standards-diluent solution before analysis.

Preparation of microtiter wells (eight- or 12-well strips or 96-well plates). We coated polystyrene white microtiter wells (MicroFluor; Dynatech Laboratories, Alexandria, VA 22314) for 18–20 h at 4 °C with, per well, 200 ng (100 μL) of purified monoclonal anti-AFP antibody (Medix Biotech Inc., Foster City, CA 94404; cat. no. A-013-01) dissolved in 50 mmol/L carbonate buffer, pH 9.6. We then washed the wells manually twice with the wash solution, added blocking solution (200 μL/well) for 1 h at room temperature, and washed the wells again, as described. Stored dry at 4 °C, they are stable for several weeks.

Biotinylation of antibody. Affinity-purified goat anti-AFP polyclonal antibody (Atlantic Antibodies, Scarborough, ME 04074; cat no. 077-06) was dialyzed twice against 5 L of isotonic saline (NaCl 9 g/L) and then diluted with carbonate buffer (0.1 mol/L, pH 9.0) to give a final concentration of 500 mg/L. To 1 mL of this solution we added a 500-fold molar excess of sulfosuccinimidyl-6-(biotinamido)hexanoate ("NHS-LC-biotin"; Pierce Chemical Co., Rockford, IL 61105) dissolved in 100 μL of dimethyl sulfoxide, mixed, and incubated for 1 h at room temperature. The reaction mixture was then dialyzed twice at 4 °C against 5 L of 0.1 mol/L bicarbonate buffer (pH 8.3) containing 0.25 g of sodium azide per liter.

Before use, we diluted the biotinylated antibody solution 300-fold in 10 mmol/L Tris HCl buffer (pH 7.8) containing, per liter, 400 mmol of KCl, 10 g of bovine serum albumin, 0.1 g of sodium azide, and 0.1 g of thimerosal.

Preparation of labeled streptavidin. After dissolving 5 mg of streptavidin (Sigma) in 33 mL of 0.1 mol/L carbonate buffer (pH 9.1), we added to this, with stirring, 7 mg of BCPDA dissolved in 200 μL of dimethylformamide, at room temperature. After 1 h, we dialyzed the reaction mixture three times against 5 L of a 0.1 mol/L solution of $NaHCO_3$ containing 0.25 g of sodium azide per liter.

Before use, we diluted the labeled streptavidin solution 50-fold in 50 mmol/L Tris HCl buffer (pH 7.8), containing, per liter, 10 g of bovine serum albumin, 9 g of NaCl, 0.1 g of sodium azide, 0.1 g of thimerosal, and Eu^{3+} to give a final concentration of 10 μmol/L.

Comparison methods. We also used a commercially available radioimmunoassay (Amersham Corp., Arlington Heights, IL 60005) and a time-resolved immunofluorometric procedure (DELFIA hAFP kit; LKB Wallac, Turku, Finland), carrying out both procedures according to the manufacturers' instructions.

Immunoassay procedure. Add 20 μL of standards or samples (duplicate or preferably triplicate measurements) to each well, then 100 μL of standards-diluent buffer. After incubating the wells for 45 min at 37 °C (air oven), wash the wells twice with the wash solution. Then add 300-fold-diluted biotinylated anti-AFP antibody solution (100 μL/well), incubate for another 45 min at 37 °C, then wash the wells as above. Add the mixed, labeled streptavidin–Eu^{3+} working solution, 100 μL per well, and incubate for 30 min more at 37 °C. Wash the wells as above and dry them with a forced-air plate dryer. Measure the fluorescence on the solid phase of each well (we used a CyberFluor 615 time-resolved fluorometer/analyzer), using an excitation wavelength of 337.1 nm (nitrogen laser source) and an emission wavelength of 615 (±5) nm (interference filter).

Results

Incubation time and temperature. We investigated the effect of the incubation time and temperature on the performance of the AFP assay. The quality of the calibration curve, the precision of the assay, and the accuracy of measurements were monitored by analyzing a series of 20 clinical samples previously assayed for AFP by RIA. We found that a precise and accurate assay could be established if the incubation times were fixed at 45, 45, and 30 min, respectively, at 37 °C. With this choice, an assay run could be completed in less than 3 h.

Sensitivity and precision. Figure 2 depicts a typical standard curve (log-log plot). The assay has a dynamic range of 1–1000 μg/L (1.03–1030 int. units/mL) and a detection limit of 0.1 μg/L, as calculated from the mean fluorescence + 3 standard deviations of the zero standard. At concentrations of analyte >1 mg/L, the curve is relatively flat and cannot be used for analytical purposes.

Precision studies were performed with Tri-level commercial control sera. As shown in Table 1, the intra-assay coefficients of variation (CVs) for AFP concentrations of 21.5, 58, and 170 ng/mL (μg/L) were 7.6, 5.4, and 6.2%, respectively. Inter-assay CVs for the same controls were 3.9, 8.6, and 1.7%, respectively. Day-to-day precision, determined over a one-month period for the same controls, was 7.2, 7.3, and 8.7%, respectively.

Recovery and linearity. To assess the recovery of the assay, analyte-suppl mented serum samples were prepared as shown in Table 2. Analytical recovery of AFP ranged from 72 to 125% (mean 104 ± 17%).

To evaluate the linearity of the assay, we tested serial dilutions of three different samples and determined the amount of AFP in each sample. The concentration of AFP decreased linearly with increasing dilution, and the values obtained were those expected, if the value of the undiluted sample is taken as a true value. This finding confirms that our assay is free of any serum matrix effects.

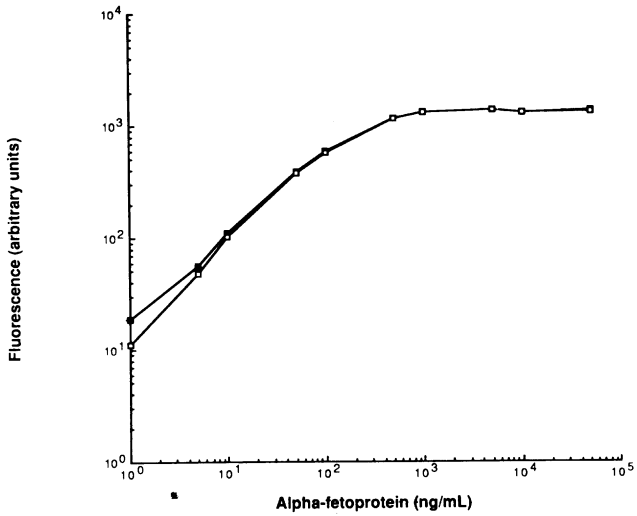

Fig. 2. AFP standard curve with (□) and without (■) background fluorescence subtracted

Table 1. Precision Studies with the Alpha-Fetoprotein Assay and Commercial Quality-Control Sera

Sample	AFP, μg/L Mean	SD	CV, %
Intra-assay (n = 21 each)			
I	21.5	1.6	7.6
II	58.0	3.1	5.4
III	170.0	10.5	6.2
Inter-assay (n = 4 each)			
I	21.0	0.8	3.9
II	68.0	5.9	8.6
III	179.0	3.0	1.7
Day-to-day			
I[a]	19.1	1.4	7.2
II	68.4	5.0	7.3
III	171.7	14.9	8.7

[a] n = 11; other two, n = 10 each

Table 2. Analytical Recovery of Alpha-Fetoprotein Added to Clinical Serum Samples

AFP, μg/L Added	Found	Recovery, %
—	20	—
100	141	121
200	245	112
—	31	—
100	156	125
200	263	116
—	19	—
100	94	75
200	266	123
—	40	—
100	127	87
200	194	77
—	7	—
100	105	99
200	199	96
—	<1	—
100	72	72
200	193	96
—	12	—
100	134	122
200	228	108
—	99	—
100	220	121
200	304	102
—	94	—
100	199	105
200	325	116

Interference studies. We studied the effect of hemolysis, lipemia, and bilirubin on the performance of the assay, by supplementing serum samples with hemoglobin, triglycerides, and bilirubin. We found no interference from these compounds at concentrations up to 10 g/L (hemoglobin), 10 g/L (triolein), and 200 mg/L (unconjugated bilirubin).

Correlation studies. A comparison study with a commercial RIA kit for 162 maternal serum and 68 amniotic fluid samples from women at various gestational ages was undertaken. The regression equations were: (*a*) for serum: y (RIA) = 1.575 + 0.905x (present method), r = 0.98; and (*b*) for amniotic fluid: y (RIA) = −0.768 + 0.986x (present method), r = 0.99. In addition, the present assay was evaluated with a commercial time-resolved fluoroimmunoassay kit (TR-FIA). When 137 serum samples were tested in both assays the regression equation was y (TR-FIA) = −14.5 + 1.30x (present method), r = 0.98.

Discussion

Although RIA and immunoradiometry are well-established analytical techniques, they have some serious disadvantages that arise from the use of radioactive nuclides as labels. For that reason there is a trend toward alternative techniques that are based on the competitive or non-competitive principle but involve either enzymes or luminescent or fluorescent molecules as labels. In the field of fluorescence immunoassay, fluorescein and other conventional probes have been used in either homogeneous or heterogeneous techniques (*13*) with success in many areas of clinical biochemistry, such as therapeutic drug monitoring. However, the use of fluorescein as a label poses some problems in immunoassays. For example, it has a very small Stokes shift (~28 nm), emission at a wavelength close to the emission wavelength of serum autofluorescence, and binding to serum components. These limitations do not allow for devising very sensitive immunoassays in the picomolar range.

Europium complexes with some organic chelates emit strong fluorescence with a number of interesting characteristics: (*a*) exceptionally large Stokes shift (~290 nm), (*b*) very sharp emission bands (band width at 50% emission ≤10 nm), (*c*) emission at a wavelength where serum autofluorescence is practically zero (615 nm), and (*d*) long-lived fluorescence (fluorescence lifetime of the order of 100–1000 μs as compared to 4.5 ns for fluorescein). A combination of these factors makes the Eu^{3+} complexes attractive labels for immunoassays in serum, because background fluorescence can be minimized by isolating the specific fluorescence at ~615 nm with a narrow-bandpass interference filter and further excluding unwanted fluorescence by applying the "time-resolved principle" in a gated fluorometer. With this principle, the sample is excited with a flash of light and measurement is carried out after a delay period during which any short-lived fluorescence has ceased. With europium complexes as fluorescent markers, the back-ground fluorescence so observed is essentially only that originating from nonspecific binding of the reagents used.

There are two general approaches to the use of europium complexes in immunoassays. In the first approach, applied successfully by Soini and Hemmila (*13–15*), europium-labeled immunoreactants are used. In the second approach, presented here, chelate-labeled immunoreactants are used. The synthesis and some spectroscopic data of the novel Eu^{3+} chelate 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid are described elsewhere (*16*). In the present system, fluorescence is quantified directly on the solid support. In the previous system, the final measurement is for Eu^{3+} in solution after its dissociation from the solid-phase. The latter approach suffers from vulnerability to Eu^{3+} contamination.

The use of the well-known streptavidin–biotin system in the assay design has a number of advantages: (*a*) It is a universal detection system in both sandwich and competitive immunoassays. (*b*) The labeling of streptavidin with the novel Eu^{3+} chelate is very easy and efficient, without any loss of biological activity. (*c*) Labeled streptavidin and biotinylated antibodies are very stable reagents. (*d*) Amplification is introduced.

In conclusion, we describe a time-resolved fluorescence immunoassay for AFP with use of a novel detection system. The capabilities of the detection system for devising other competitive and non-competitive immunoassays are described elsewhere (*17*).

We thank Dr. P. Y. Wong of the Toronto General Hospital for providing the clinical samples and for stimulating discussions.

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