Enzymatically Amplified Time-Resolved Fluorescence Immunoassay with Terbium Chelates

Theodore K. Christopoulos and Eleftherios P. Diamandis*

Department of Clinical Biochemistry, Toronto Western Hospital, 399 Bathurst Street, Toronto, Ontario M5T 2S8, Canada, and Department of Clinical Biochemistry, University of Toronto, 100 College Street, Toronto, Ontario M5G 1L5, Canada

We report an ultrasensitive, enzymatically amplified, time-resolved fluorescence immunoassay with a terbium chelate as the detectable moiety. In this immunoassay, the primary label is the enzyme alkaline phosphatase (ALP). ALP cleaves phosphate out of a fluorogenic substrate, 5-fluorosalicyl phosphate, to produce 5-fluorosalicylic acid (FSA). 5-Fluorosalicylic acid can then form a highly fluorescent ternary complex of the form FSA–Tb^{3+}–EDTA, which can be quantified by measuring the Tb^{3+} fluorescence in a time-resolved mode. In this assay, exceptional sensitivity is achieved because of the enzymatic amplification introduced by ALP and the quantification by laser-induced microsecond time-resolved fluorometry. Time-resolved fluorometry is applicable because of the long fluorescence lifetime of the Tb^{3+} complexes. It is shown that in a model AFP assay 10^6 or 1.5×10^5 molecules can be detected (final assay volume, 100 μL) by using monoclonal or polyclonal detection antibodies, respectively. The assay demonstrates excellent precision ($\sim$4%), and it seems to be highly suited for automated, sensitive, and rapid immunoassays.

INTRODUCTION

Among the nonisotopic immunoassay methodologies, time-resolved fluorescence immunoassay is well-established and successful. The commercially available systems are based on the use of fluorescent europium chelates as labels, and the technology has been recently reviewed.[1–5] The main advantages of the fluorescent europium chelates have been repeatedly stressed. These are also shared with other lanthanide chelates and especially with the fluorescent Tb^{3+} and Sm^{3+} chelates. Such advantages include large Stokes shifts, narrow emission bands, and long fluorescence lifetimes. The long lifetime makes these chelates especially suitable as labels for microsecond time-resolved fluorescence immunoassay.

Europium has been, up to now, the lanthanide label of choice for time-resolved fluorescence immunoassay because of its superior detectability in comparison to Tb^{3+} and more so to Sm^{3+}.[6] The last two lanthanides have primarily been used as comparison labels to devise dual analyte assays as described elsewhere.[7–10] Tb^{3+} alone has been proposed as a primary immunological or nucleic acid label mainly because of its ability to form ternary fluorescent mixed complexes with aminopolycarboxylic acids (e.g. EDTA or DTPA) and certain ligands like *p*-aminosalicylate.[11–15] However, the better sensitivity of the Eu^{3+}-assayed systems precluded the widespread use of these complexes.

Tb^{3+} complexes, however, have some advantages over the Eu^{3+} complexes. Tb^{3+} can form fluorescent complexes with a wider variety of organic ligands, many of these complexes are freely soluble in water, and quenching by coordinated water molecules is not a serious problem. On the other hand, in ternary Tb^{3+} complexes, slight modification of the coordinated ligand, especially on the hydroxyl which is adjacent to the carboxyl group of the ligand (e.g. in salicylate), usually abolishes or dramatically diminishes its ability to coordinate and thus to form fluorescent complexes. So, salicylate or similar compounds can be modified to become enzyme substrates as further exemplified below. The ternary Tb^{3+} complexes are usually fluorescent at strongly alkaline pH, i.e. 12–13. At this pH, the aminopolycarboxylic acid ligand, e.g. EDTA, serves to chelate Tb^{3+} with high affinity and keeps it soluble in water without the danger of hydroxide salt precipitation. Ligands like salicylate coordinate around Tb^{3+} to form the ternary complex. After ligand excitation, the energy absorbed by the ligand is transferred to Tb^{3+} by an internal energy transfer process.[1] Tb^{3+} is then excited and subsequently fluoresce at characteristic wavelengths. The ternary complex between DTPA, Tb^{3+}, and *p*-aminosalicylate (pAS)

* To whom correspondence should be addressed at the Toronto Western Hospital.

0003-2700/92/0364-0342$03.00/0 © 1992 American Chemical Society

can also be created by covalently linking DTPA and pAS as described elsewhere.[14]

When salicylate or derivatives are modified at the hydroxyl group to e.g. phosphate esters, they can no longer coordinate efficiently around the EDTA–Tb^{3+} complex and the fluorescent complex is not formed. The complex can form in the presence of an enzyme, i.e. alkaline phosphatase, which can split the phosphate ester.

Recently, the phosphate ester of 5-fluorosalicylate and other salicylate derivatives have been synthesized and proposed as substrates for alkaline phosphatase and other enzymes.[16] We here report the application of the alkaline phosphatase substrate for devising highly sensitive time-resolved fluorometric immunoassays.

EXPERIMENTAL SECTION

Materials. The phosphate ester of 5-fluorosalicylic acid (FSAP) from the REALL-S detection kit for alkaline phosphatase was a gift from A. Pollak, Kronem Systems Inc., Mississauga, Ontario, Canada. It was supplied as a solid reagent (4.7 mg/vial) and was stored dry at −20 °C until use. 5-Fluorosalicylic acid (FSA) was purchased from Aldrich, Milwaukee, WI. Calf intestine alkaline phosphatase (ALP) was obtained from Boehringer Mannheim, Montreal, PQ. Alkaline phosphatase labeled streptavidin (SA-ALP) was obtained from Zymed Laboratories, Inc., San Francisco, CA, as a 0.75 mg/mL solution. White, opaque 12-well polystyrene microtiter strips without any coating or coated with a monoclonal anti-α-fetoprotein (AFP) antibody were obtained from CyberFluor Inc., Toronto, Ontario. The monoclonal and polyclonal (affinity purified) detection antibodies used for the AFP immunoassay are available from CyberFluor. $TbCl_3 \cdot 6H_2O$ was from GFS Chemicals, Columbus, OH. All other chemicals used were from Sigma Chemical Co., St. Louis, MO, unless otherwise stated.

Solutions. We used two different substrate buffers, widely recommended in the literature for alkaline phosphatase assays: (a) Tris buffer, 0.1 mol/L, pH 9.0, containing 0.1 mol of NaCl and 1 mmol of $MgCl_2$ per liter; (b) diethanolamine buffer, 0.95 mol/L, pH 9.8, containing 1 mmol of $MgCl_2$ per liter.

A stock 10^{-3} mol/L 5-fluorosalicylic acid solution was prepared by dissolving 3.12 mg of FSA in 2 mL of dimethyl sulfoxide. More dilute FSA solutions were prepared by dilution in the substrate buffer. A stock 10^{-2} mol/L 5-fluorosalicylic acid phosphate ester solution was prepared by dissolving the contents of one vial (4.7 mg) in 2 mL of a 0.1 mol/L NaOH solution. This solution was stored at 4 °C up to a period of 2 weeks. Fresh FSAP substrate working solutions were prepared just before use by dilution (10-fold) of the stock in the substrate buffer (a or b) described above.

A "general diluent" was a 6% (w/v) solution of bovine serum albumin in a 50 mmol/L Tris buffer, pH 7.40, containing 0.5 g of sodium azide per liter.

The biotinylation of the monoclonal and polyclonal detection antibodies was performed by using sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-Biotin) as previously described.[17] The stock solutions of the biotinylated antibodies were 0.66 mg/mL (monoclonal) and 0.22 mg/mL (polyclonal). Working solutions were prepared by dilution in the general diluent as described under Methods.

Solutions of ALP of various concentrations were prepared by diluting the 10 mg/mL stock solution in the substrate buffer. A working solution of the streptavidin–alkaline phosphatase conjugate was prepared by diluting the stock solution 5000-fold in a 50 mM Tris buffer, pH 7.40, containing 3% (w/v) of BSA.

The wash solution was prepared by dissolving 9 g of NaCl and 0.5 mL of polyoxyethylenesorbitan monolaureate (Tween 20) in 1 L of distilled water.

α-Fetoprotein standards in the range of 0–200 ng/mL were obtained from CyberFluor. Standards with very low AFP levels were prepared by freshly diluting the 5 ng/mL standard with the general diluent.

The "developing solution" is a Tb^{3+}–EDTA containing reagent and is prepared just before use by mixing 1 part of a 5×10^{-3} mol/L Tb^{3+}–EDTA solution in 0.01 M HCl, 1 part of a 2.5 M Tris buffer of pH 13.0, and 3 parts of water. The final composition of the working developing solution is 10^{-3} mol/L of the Tb^{3+}–EDTA complex and 0.5 mol/L Tris.

Instrumentation. For measuring liquid-phase Tb^{3+} fluorescence in white microtiter wells we used the Cyberfluor 615 immunoanalyzer, a time-resolved fluorometer. The time-gate settings of the instrument as described in ref 1 were not changed because the fluorescence lifetimes of the ternary complexes of Tb^{3+} with EDTA and salicylate derivatives are not very different from those of the fluorescent Eu^{3+} complexes.[1,11,14] The interference filter of the emission pathway was also retained (615 nm) for reasons described under Results and Discussion. During the optimization experiments, we also tried filters with nominal wavelengths of 488.0, 545.0, and 593.0 nm, purchased from SpectroFilm (Woburn, MA).

Methods. Determination of FSA with Tb^{3+}–EDTA and Time-Resolved Fluorometry. Pipet 100 μL of FSA solution in substrate buffer in the concentration range of 10^{-9}–10^{-5} mol/L into noncoated white microtiter wells. Then add 100 μL of Tb^{3+}–EDTA working developing solution, mix by mechanical shaking for 1 min, and measure the fluorescence with the time-resolved fluorometer.

Determination of Alkaline Phosphatase Using 5-Fluorosalicylic Acid Phosphate Ester (FSAP) as Substrate. The protein-binding sites of the noncoated microtiter wells were first blocked by incubation with 200 μL of the general diluent for 1 h, followed by washing four times with the wash solution. This pretreatment was done to avoid possible adsorption of ALP to the plastic. We then pipetted 50 μL of ALP solution (various dilutions) and added 50 μL of a 2×10^{-3} mol/L working FSAP substrate solution. After incubation for 30 min at room temperature (RT), we added 100 μL of the working developing solution (Tb^{3+}–EDTA), mixed by mechanical shaking for 1 min, and measured the fluorescence with the time-resolved fluorometer.

One-Step AFP Assay with Monoclonal Detection Antibody: Protocol A. A set of AFP standards of concentrations 0, 0.2, 2, 20, and 400 ng/mL was used for this protocol. The above standards and serum samples must be diluted 10-fold in the general diluent before assay. Pipet 50 μL of diluted standard or serum samples into monoclonal antibody coated wells (in duplicate) and then add 50 mL of biotinylated monoclonal detection antibody diluted 1600-fold in the general diluent. Incubate with mechanical shaking for 90 min at room temperature and then wash four times. Add 100 μL/well of the streptavidin–alkaline phosphatase conjugate, incubate 15 min as above, and wash four times. Add 100 μL/well of the 10^{-3} mol/L working FSAP substrate solution and incubate for 15 min as above. Then add 100 μL of the working developing solution (Tb^{3+}–EDTA), mix by mechanical shaking for 1 min, and measure the fluorescence with the time-resolved fluorometer. The calibration curve and data reduction are carried out automatically by the analyzer.

Rapid One-Step AFP Assay with Monoclonal Detection Antibody: Protocol B. AFP standards of concentrations 0, 1, 5, 10, 25, 50, 100, and 200 ng/mL are used undiluted in this protocol. Pipet 25 μL of the standards or undiluted samples in each well (in duplicate) and then add 75 μL of biotinylated monoclonal detection antibody diluted 400-fold in the general diluent. The assay is then completed exactly as described under protocol A, but the three incubation times of 90, 15, and 15 min are replaced by 5, 5, and 5 min, respectively.

Two-Step AFP Assay with Polyclonal Detection Antibody: Protocol C. For this assay, we used AFP standards of concentrations 0, 1, 5, 20, 100, and 500 pg/mL. Pipet 100 mL of standards into monoclonal antibody coated wells (in duplicate), incubate for 2 h at RT with mechanical shaking, and wash two times. Add 100 μL/well of biotinylated polyclonal detection antibody diluted 200-fold in the general diluent. Incubate with mechanical shaking for 60 min at RT and wash four times. Complete the assay exactly as in protocol A from the point of adding the streptavidin–alkaline phosphatase conjugate but incubate the FSAP substrate solution for 30 min instead of 15 min.

RESULTS AND DISCUSSION

The dephosphorylation reaction of FSAP by ALP and the complexation of FSA with Tb^{3+}–EDTA to form the fluorescent complex is shown in Figure 1. The fluorescent complex has an excitation maximum at 336 nm (characteristic of FSA) and

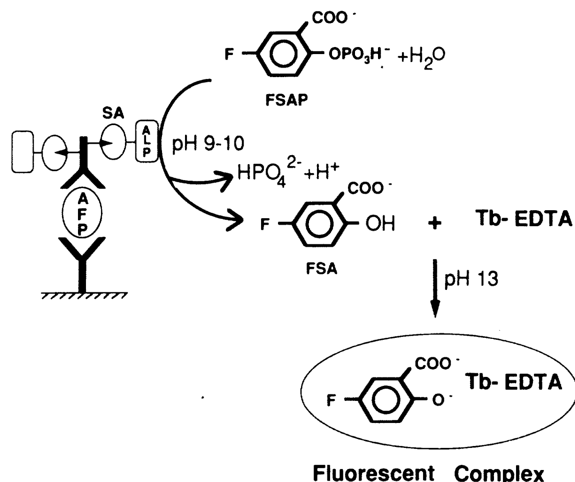

Figure 1. Principle of Tb^{3+}-chelate-based enzymatically amplified time-resolved fluorescence immunoassays. The analyte (AFP) is captured by the coating antibody. A "sandwich" is formed by adding a biotinylated detection antibody, followed by the addition of alkaline phosphatase (ALP) labeled streptavidin (SA). ALP hydrolyzes the phosphate ester of fluorosalicylic acid (FSAP) to give free fluorosalicylic acid (FSA) which is then quantitated by adding a Tb^{3+}–EDTA solution and measuring the fluorescence of Tb^{3+} in the mixed complex, with a time-resolved fluorometer.

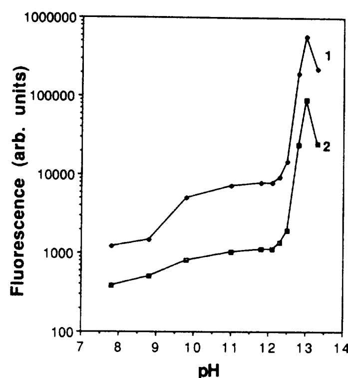

Figure 2. Effect of pH on the fluorescence of FSA–Tb^{3+}–EDTA solutions at FSA concentrations: (1) 5×10^{-6} and (2) 5×10^{-7} M. Tb^{3+}–EDTA concentration is 5×10^{-4} M. 1 M Tris buffers were used throughout this study.

emission maxima at 491, 548, 587, and 624 nm (characteristic of the metal ion).

The measurement of Tb^{3+} fluorescence was accomplished by using the unmodified 615 immunoanalyzer (a time-resolved fluorometer) briefly described in ref 1. This instrument has a nitrogen laser as an excitation source (337.1 nm). The emission pathway has an interference filter with a nominal wavelength of 615 nm, the emission maximum of Eu^{3+}. We preferred to use the unmodified instrument because Tb^{3+} fluorescence could be monitored at 615 nm, a wavelength close to the 624 nm peak of Tb^{3+}. When we changed the filter and replaced it with one with a nominal wavelength of 545.0 nm (or the other Tb^{3+}-characteristic wavelengths), we noticed a large increase in the obtained signals in the immunological assay for AFP but also a parallel increase in the background signal. Background signal is presumably due to Tb^{3+}–EDTA interaction with FSAP. Overall, the signal to background ratio was similar at any of the five emission wavelengths tested.

The effect of pH on the fluorescence of FSA–Tb^{3+}–EDTA solutions at FSA levels of 5×10^{-7} and 5×10^{-6} mol/L is shown in Figure 2. Maximum fluorescence is obtained at pH 13. At other pH levels the fluorescence is much decreased. A calibration curve for measuring FSA in the presence of excess Tb^{3+}–EDTA at pH 13 is shown in Figure 3. The detection

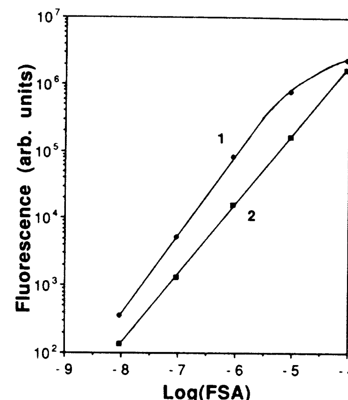

Figure 3. Quantification of 5-fluorosalicylic acid (FSA) after complexation with Tb–EDTA. FSA was dissolved in either a Tris-based (1) or diethanolamine-based (2) substrate buffer. The fluorescent immunocomplex was formed by the addition of the Tb–EDTA developing solution of pH 13.0.

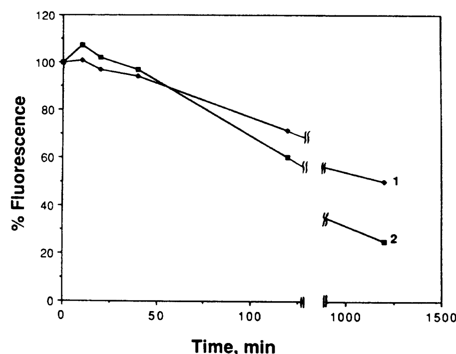

Figure 4. Stability of the fluorescence of FSA–Tb^{3+}–EDTA solutions. Percent of the fluorescence remained vs time at two FSA levels: (1) 9×10^{-6} and (2) 9×10^{-7} M.

limit, defined as the FSA concentration that produces a signal which is equal to the background signal plus two standard deviations of the background signal, is 5×10^{-9} mol/L.

We further studied the stability of the fluorescence of FSA–Tb^{3+}–EDTA solutions with time. Results are shown in Figure 4. The fluorescence decreases by about 10% and 40% in 1 and 2 h, respectively. We found that the major contributor of this decrease is the gradual drop in pH which is due to CO_2 absorption. If the same solutions are kept for the above time intervals under a nitrogen atmosphere, the drop of fluorescence is prevented.

Many alkaline phosphatase substrate solutions hydrolyze slowly with time without the presence of the enzyme. This is a disadvantage because of the increase in signal backgrounds. In order to study the spontaneous hydrolysis of FSAP, we incubated at room temperature 100 μL of the working substrate solution for 0, 30, 60, 120, and 240 min; we then added the Tb^{3+}–EDTA working developing solution and measured the resulting fluorescence. We observed a 2-fold increase in the signal after 240-min incubation in comparison to the zero time signal. In the assays mentioned later all substrate incubations were kept at 30 min so that the spontaneous substrate dephosphorylation effect is not significant. The same experiment was repeated with use of the diethanolamine substrate buffer, and we found no significant difference in fluorescence between the zero and 240-min substrate incubation. Although the diethanolamine buffer is advantageous in this respect, we preferred to routinely use the Tris-based substrate buffer because it gives better detection limits as mentioned below.

We have constructed calibration curves for the determination of alkaline phosphatase in solution using either Tris

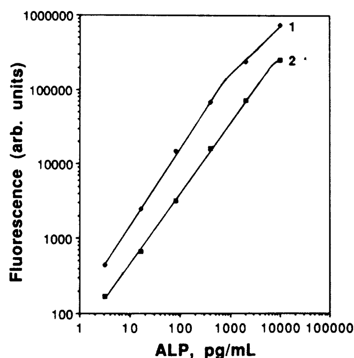

Figure 5. Quantification of alkaline phosphatase (ALP) solutions in either a Tris-based buffer (1) or diethanolamine buffer (2). ALP catalyzes the dephosphorylation of FSAP and the FSA produced is reacted with the Tb-EDTA complex to form the fluorescent complex. For details see Methods. The detection limit is 1.0 and 2.8 pg/mL for curves 1 and 2, respectively.

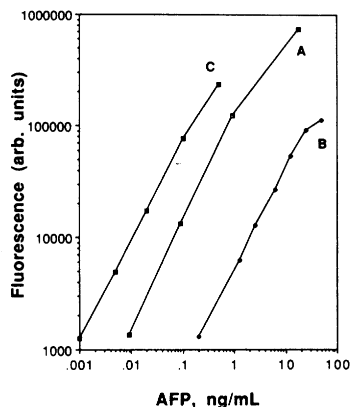

Figure 6. Typical calibration curves for AFP assay with various protocols. (A) One-step assay with monoclonal detection antibody (protocol A); (B) rapid one-step AFP assay with monoclonal detection antibody (protocol B); (C) two-step AFP assay with polyclonal detection antibody (protocol C).

or diethanolamine substrate buffer (Figure 5). The calculated detection limits in Tris and diethanolamine buffers were found to be 1.0 and 2.8 pg/mL, respectively. The 1 pg/mL concentration ($\sim 1.2 \times 10^{-14}$ mol/L) corresponds to 0.6 amol or 360 000 alkaline phosphatase molecules in a sample volume of 50 μL. Precision studies revealed that the within-run coefficients of variation of quantifying alkaline phosphatase concentration with the above method are between 1 and 2% in the whole range of measurements. Because of the superior sensitivity obtained with the Tris substrate buffer, it was selected for all further studies.

A typical calibration curve for the determination of α-fetoprotein by the one-step immunological assay (protocol A) is shown in Figure 6A. The detection limit of this assay is 1.0 pg/mL of AFP (final concentration in the reaction mixture). The dynamic range extends from the detection limit to 20 ng/mL. The accuracy of this method was assessed by analyzing (after appropriate dilution) 30 serum samples with AFP concentrations in the range 5–136 ng/mL by this and another widely used automated AFP procedure (the Abbott IMx method). The linear regression equation was y (proposed method) = $-4.8 + 0.95$(IMx) with a correlation coefficient equal to 0.99 (Figure 7).

We have devised protocol B in order to examine the possibility of completing the AFP assay in a total time of 15 min. A typical calibration curve with this rapid protocol is shown in Figure 6B. The detection limit calculated was 10 pg/mL, a value $\sim$10-fold greater than the detection limit of protocol A. Nevertheless, the reference range for AFP in human serum is 500–9000 pg/mL;[18] so, protocol B still has more than adequate sensitivity for practical clinical applications. The results with protocol B clearly suggest that FSAP, when used as substrate as described, can support highly sensitive and rapid immunological assays and may be used as substrate in fully automated clinical immunoanalyzers. This possibility is currently under investigation.

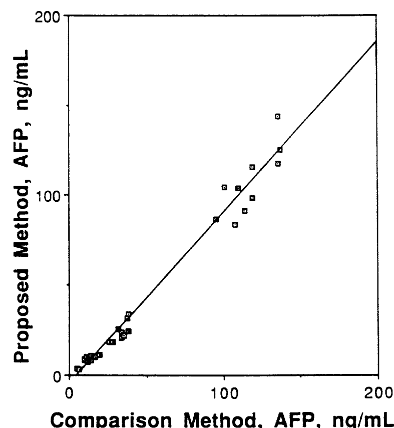

Figure 7. Linear regression analysis of results obtained by the proposed (y) and a widely accepted method (x) for AFP analysis in serum. The comparison method is a fluorometric immunoassay automated in the IMx analyzer.

We have previously observed that biotinylated polyclonal detection antibodies for AFP can achieve better detection limits than biotinylated monoclonal detection antibodies.[18] We have established another protocol for AFP assay (protocol C) which uses a biotinylated polyclonal detection antibody. Here, our aim was the highest sensitivity achievable with the proposed detection system. A typical calibration curve is shown in Figure 6C. The detection limit was 0.15 pg/mL of AFP (2.5×10^{-15} mol/L) which corresponds to $\sim$150 000 molecules of AFP in a sample volume of 100 μL. Precision studies with all three protocols at various AFP level gave CV's of $\leq$4% in all cases.

This report deals with enzymatically amplified time-resolved fluorometric immunoassays. The method combines the advantages of lanthanide chelates as fluorometric labels and the ability of enzymes and the biotin–avidin system to amplify the signal. With this method it is possible to quantify analytes at extremely low levels (i.e. down to $\sim$150 000 molecules). Alternatively, it would be possible to devise sensitive and very quick assays suitable for routine use.

We believe that the full potential of this detection system can be realized when used as part of a fully automated devise which will combine high-speed analysis and high throughput of samples.

ACKNOWLEDGMENT

We thank Alfred Pollak, Kronem Systems Inc., for the generous supply of the FSAP substrate and for helpful discussions.

REFERENCES

(1) Diamandis, E. P. *Clin. Biochem.* **1988**, *21*, 139–150.
(2) Diamandis, E. P. *Clin. Chim. Acta* **1990**, *194*, 19–50.
(3) Diamandis, E. P.; Christopoulos, T. K. *Anal. Chem.* **1990**, *62*, 1149A–1157A.
(4) Soini, E.; Lovgren, T. *CRC Crit. Rev. Anal. Chem.* **1987**, *18*, 105–154.
(5) Soini, E.; Hemmila, I.; Dahlen, P. *Ann. Biol. Clin.* **1990**, *48*, 567–571.
(6) Hemmila, I. *Scand. J. Clin. Lab. Invest.* **1988**, *48*, 389–400.
(7) Hemmila, I.; Holttinen, S.; Pettersson, K.; Lovgren, T. *Clin. Chem.* **1987**, *33*, 2281–2283.
(8) Siitari, H. *J. Virol. Methods* **1990**, *28*, 179–188.
(9) Bador, R.; Dechaud, H.; Claustrat, F.; Desuzinges, C. *Clin. Chem.* **1987**, *33*, 48–51.
(10) Saarma, M.; Jarvekulg, L.; Hemmila, I.; Siitari, H.; Sinijaru, R. *J. Virol. Methods* **1989**, *23*, 47–54.
(11) Bailey, M. P.; Rocks, B. F.; Riley, C. *Analyst* **1984**, *109*, 1449–1450.
(12) Hemmila, I. *Anal. Chem.* **1985**, *57*, 1676–1681.

(13) Kuo, J. E.; Milby, K. H.; Hinsberg, W. D.; Poole, P. R.; McGuffin, V. L.; Zare, R. N. *Clin. Chem.* **1985**, *31*, 50–53.
(14) Bailey, M. P.; Rocks, B. F.; Riley, C. In *Non-Isotopic Immunoassay*; Ngo, T. T., Ed.; Plenum Press: New York, 1988; pp 187–197.
(15) Oser, A.; Collasius, M.; Valet, G. *Anal. Biochem.* **1990**, *191*, 295–301.
(16) Evangelista, R. A.; Pollak, A.; Gudgin-Templeton, E. F. *Anal. Biochem.* **1991**, *197*, 213–224.
(17) Chan, M. A.; Bellem, A. C.; Diamandis, E. P. *Clin. Chem.* **1987**, *33*, 2000–2003.
(18) Christopoulos, T. K.; Lianidou, E. S.; Diamandis, E. P. *Clin. Chem.* **1990**, *36*, 1497–1502.

RECEIVED for review July 12, 1991. Accepted November 11, 1991.

0003-2700/92/0364-0346$03.00/0 © 1992 American Chemical Society