Clin Biochem, Vol. 21, pp. 291–296, 1988
Printed in Canada.

0009–9120/88 $3.00 + .00

Time-Resolved Fluoroimmunoassay of Cortisol in Serum with a Europium Chelate as Label

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A non-isotopic heterogeneous competitive immunoassay of serum cortisol is described. Cortisol present in the sample competes with immobilised cortisol (cortisol-thyroglobulin conjugate) for binding to a monoclonal anti-cortisol biotinylated antibody. The amount of antibody bound is measured on the dry solid-phase by time-resolved fluorometry after adding streptavidin labeled with the Eu^{3+} chelate 4,7-bis(chlorosulfophenyl)-1,10 phenanthroline-2,9-dicarboxylic acid (BCPDA), in the presence of excess Eu^{3+}. The assay is simple to perform, its characteristics are similar to those of radioimmunoassay techniques, and is suitable for routine clinical use.

KEY WORDS: europium; streptavidin-biotin; non-isotopic immunoassay; fluorescent labels; time-resolved fluorescence.

Introduction

Time-resolved fluorometry is now becoming an established analytical technique in the field of non-isotopic immunoassay. A complete analytical system for time-resolved fluorometric immunoassays consists mainly of a suitable instrument/analyzer and a working chemical system. Recently, we have synthesized a new europium chelate, 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) which can be used for protein labeling and time-resolved fluorometric applications (1). An instrument is now commercially available for laser-excited solid-phase time-resolved fluorometric measurements. This instrument is briefly described elsewhere (2). The combination of appropriate compounds (*e.g.*, streptavidin) labeled with BCPDA and other auxiliary reagents (*e.g.*, biotinylated antibodies) with the analyzer results in an integrated analytical system which can be used routinely for performing non-isotopic immunoassays. This new system has recently been reviewed (2) and applied successfully to the immunofluorometric assays of alpha-fetoprotein (3) and choriogonadotropin (4) in serum. In this paper, a serum cortisol assay is described using BCPDA as label. In the assay, serum cortisol competes with immobilised cortisol (cortisol-thyroglobulin conjugate adsorbed on white microtitration wells) for binding to a soluble biotinylated monoclonal anti-cortisol antibody. After washing, the degree of binding of the biotinylated antibody to the solid-phase, which is inversely related to the cortisol concentration in the sample, is quantitated by a bridge reaction with BCPDA-labeled streptavidin in the presence of excess Eu^{3+}. The fluorescent complex formed (consisting of thyroglobulin-cortisol-antibody-biotin-streptavidin-BCPDA-Eu^{3+}) is measured on the dry solid-phase by pulsed excitation with a nitrogen laser (at 337.1 nm) and monitoring the specific Eu^{3+} fluorescence at about 615 nm during a preselected time window (time-resolved measurement). The characteristics of the new assay are similar to those of other immunological cortisol assays which are currently commercially available.

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Manuscript received December 17, 1987; revised February 23, 1988; accepted February 29, 1988.

Materials and methods

Instrumentation

For solid-phase time-resolved fluorometric measurements we used the CyberFluor 615™ Immunoanalyzer. Radioactivity counting was performed with the LKB Wallac 1275 Minigamma counter.

Chemicals and solutions

The europium chelate 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) was synthesized as described in (1). Streptavidin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, MO 63178. $EuCl_3$ hexahydrate was from Aldrich Chemical Co., Milwaukee, WI 53201. Sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin) was from Pierce Chemical Co., Rockford, IL 61105. All other chemicals were from Sigma. White opaque 12-well microtiter strips were products of Dynatech Labs, Alexandria, VA 22314.

The coating buffer was a 0.1 mol/L carbonate solution of pH 9.5. The blocking buffer was a 50 mmol/L sodium phosphate solution of pH 7.4 containing 9 g NaCl, 1 g gelatin and 1 mL polyoxyethylenesorbitan monolaurate (Tween 20) per liter. The cortisol assay buffer was a 50 mmol/L Tris-HCl solution of pH 7.8 containing 65 g trichloroacetic acid, 10 g BSA, 9 g NaCl and

0.5 g sodium azide per liter. The streptavidin-europium dilution buffer was a 50 mmol/L Tris-HCl solution of pH 7.8 containing 9 g NaCl, 10 g BSA and 0.5 g sodium azide per liter. The wash solution was a 9 g/L NaCl solution containing 0.5 mL Tween 20 per liter.

Cortisol standards were prepared in cortisol-free serum. Cortisol-free serum was prepared as described by Chard (5). The cortisol monoclonal antibody was stored as a 1 mg/mL solution in 0.1 mol/L phosphate buffer of pH 7.40. A 1×10^{-3} mol/L $EuCl_3$ stock solution was prepared in 0.01 mol/L HCl.

Monoclonal antibody

The cortisol monoclonal antibody was purchased as a 1 mg/mL solution from Medix Biotech Inc., Foster City, CA 94404.

COMPARATIVE METHODS

For comparison studies we have used the Coat-A-Count Cortisol ^{125}I RIA (Diagnostic Products Corp., Los Angeles, CA 90045) and the Gamma Coat ^{125}I RIA kit (Dade, Cambridge, MA 02139)

LABELING OF STREPTAVIDIN WITH BCPDA

Affinity purified streptavidin (Sigma) is dissolved in 0.1 mol/L carbonate/bicarbonate buffer, pH 9.1, to obtain a concentration of 0.15 mg/mL. To an aliquot of this preparation is then added a 50-fold molar excess of BCPDA dissolved in a small volume (<10% of total reaction volume) of ethanol. After 1 h incubation at room temperature, the mixture is dialyzed against three changes of 0.1 mol/L sodium bicarbonate solution, pH 8.3, containing 0.5 g of sodium azide per liter. This stock preparation of the labeled streptavidin is stored at 4°C. The indicator reagent is prepared by diluting the stock preparations of BCPDA labeled streptavidin (50-fold) and of europium (100-fold) with the streptavidin-europium dilution buffer. This indicator reagent contains 3 μg/mL BCPDA labeled streptavidin and 10^{-5} mol/L $EuCl_3$. It is stable for several months when stored in an amber bottle at 4°C.

BIOTINYLATION OF CORTISOL MONOCLONAL ANTIBODY

The antibody solution (1 mL, containing 1 mg antibody) was dialyzed twice in 5 L of a 9 g/L NaCl solution at 4°C and then diluted 1:1 with a 0.5 mol/L carbonate buffer of pH 9.1. The solution was then concentrated to 0.5 mL by centrifugation in a Centricon™ 30 microconcentrator (Amicon Canada, Oakville, Ontario L6H 2B9). To this solution was added 2 mg of NHS-LC-biotin dissolved in 50 μL dimethyl sulfoxide and the mixture incubated for 2 h at room temp. The solution was then dialyzed twice in 5 L of a 9 g/L NaCl solution at 4°C and its volume adjusted to 1.0 mL with the same solution. For the assay, the antibody solution is diluted 200-fold in the assay buffer (working antibody solution). The stock antibody solution is stable for at least six months and the working antibody solution for at least one week at 4°C.

PREPARATION OF THYROGLOBULIN-CORTISOL CONJUGATE

The procedure is a modification of the mixed anhydride method (6) and it was carried out as described by Elder *et al.* (7) with the modification that cortisol 21-hemisuccinate derivative was used instead of progesterone-3-o-carboxymethyloxime. The final product, after exhaustive dialysis, was assayed for total protein with the Bio-Rad protein assay and stored at 4°C as a 3–6 mg/mL solution. It is stable for at least six months.

COATING OF MICROTITER STRIPS

The strips were coated overnight at room temperature with 100μL of a 6 μg/mL solution of cortisol-thyroglobulin conjugate in the coating buffer. After coating, the plates were rinsed once with the wash solution, blocked for 1 h at room temperature with 200 μL of the blocking buffer, washed twice and air dried overnight at room temperature. When stored in sealed plastic bags at 4°C with dessicant, they are stable for at least six months.

ASSAY PROCEDURE

Before the assay, the strips are washed twice with the wash solution. Ten μL of standard or serum samples (in duplicates) are pipetted into each well and 100 μL of the working biotinylated antibody solution added. The strips are then briefly shaken (1 min) in an automatic shaking device and incubated for 1 h at 37°C (air-oven). The strips are then washed three times with the wash solution and 100 μL of the working indicator reagent (streptavidin-BCPDA-Eu^{3+}) added. After incubation for 30 min at 37°C, the strips are washed three times with the wash solution and dried with a stream of air. Surface fluorescence is measured on a CyberFluor 615 Immunoanalyzer. The instrument has an automatic data reduction capability, and results along with the calibration curve are printed automatically as soon as the readings are complete.

Results

ASSAY OPTIMIZATION AND PERFORMANCE

We have constructed calibration curves with varying amounts of thyroglobulin-cortisol conjugate added to the wells during coating (between 70 and 1200 ng/well). We observed that at coatings higher than 500 ng the capacity of the well is saturated (no further increase in B_0, see below). The shape of the calibration curve ($(B/B_0 \times 100)$ vs. log [cortisol]) does not change significantly at coatings between 70–500 ng/well. However, the B_0 value (fluorescence obtained with the zero standard) increases with increasing the amount of coating up to the saturation point. For that reason, a coating

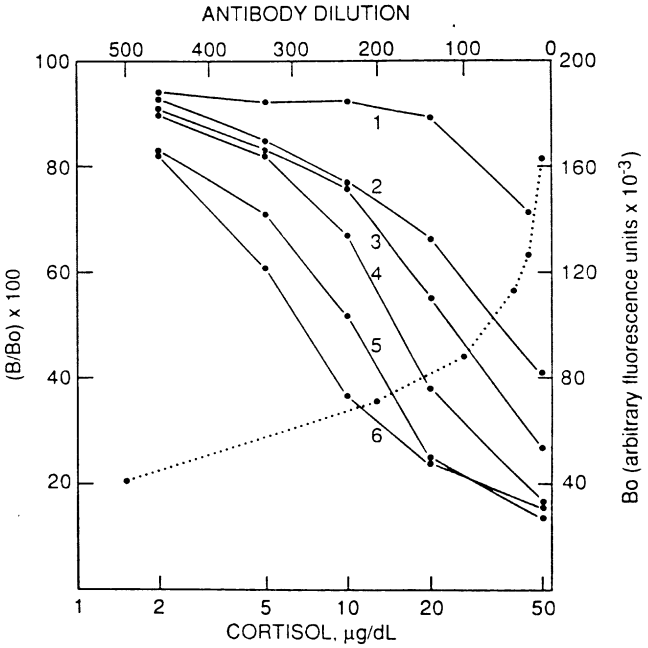

Figure 1—Calibration curves of the cortisol assay with various dilutions of the biotinylated antibody (initial concentration 1 mg/mL). (1) 10-fold; (2) 30-fold; (3) 50-fold; (4) 100-fold; (5) 200-fold; (6) 500-fold. For the assay, curve 5 is used. The value of B_0 increases with increasing the amount of antibody added (. . . . .).

of 600 ng/well was selected in order to achieve the highest value of B_0.

The effect of biotinylated antibody dilution and sample volume on the shape of the calibration curve was also studied. We observed, as expected, that the sensitivity of the calibration curve increases with increasing dilution of antibody (Figure 1) or increasing the sample volume. The most favourable combination of antibody dilution and sample volume was selected and was 200-fold and 10 µL, respectively. With this combination, the calibration curve was sufficiently sensitive (detection limit of at least 1 µg/dL cortisol) and analytically useful up to at least 50 µg/dL cortisol. The B_0 value increases with increasing amount of biotinylated antibody added (Figure 1).

We have studied the effect of incubation times on the assay performance. The antibody binding to the solid-phase (first incubation step) was increased by increasing the incubation time from 10 to 120 min at 37°C, but the rate of increase was much lower after the first 60 min of incubation. For this reason, we have chosen the first incubation to be 60 min at 37°C. We have also studied the time-course of labeled streptavidin binding (second incubation step) between 10 and 80 min, at 37°C. Streptavidin binding increased continuously during this time period, but the rate of binding was much lower after the first 30 min. A second incubation time of 30 min was thus chosen as a compromise.

A typical calibration curve for the assay is shown in Figure 1 (curve 5). The detection limit of the assay calculated from the point which is 2SD of the zero standard below the response of the zero standard is ≤1 µg/dL.

The precision of the assay is shown in Table 1. The recovery of added cortisol is shown in Table 2. The cross-reactivity of the monoclonal antibody used with a number of steroids or drugs, calculated as the ratio of cortisol concentration to the cross-reacting substance concentration at 50% inhibition of maximum binding, expressed as a percentage, was as follows: cortisol (100), cortisone (35), prednisolone (40), prednisone (30), corticosterone (30), 11-deoxycortisol (12), 11-dehydrocorticosterone (4), deoxycorticosterone (4), 17-α-hydroxyprogesterone (2), progesterone (<1), dexamethasone (<1), and spironolactone (<1).

The comparison of results between the proposed method and two widely used radioimmunoassay procedures is shown in Figure 2. Regression equations were: y (present method) = −0.203 + 0.976 × (DPC, RIA), r = 0.96; n = 99 and y (present method) = 0.316 + 1.000 × (Dade, RIA), r = 0.95; n = 65. The linearity of the assay was established by analyzing three different samples with high cortisol concentrations at varying dilutions. The results are shown in Figure 3.

TABLE 1
Precision of the Cortisol Assay

Sample	Cortisol, µg/dL Mean	S.D.	%CV	N
Within-run				
A	8.7	0.4	4.2	12
B	16.5	0.8	5.0	12
C	30.0	1.8	5.9	12
Day-to-Day*				
D	10.7	0.8	7.3	9
E	18.2	1.6	8.9	9
F	40.4	3.3	8.2	9

*Over a period of one month.

TABLE 2
Recovery of Added Cortisol to Serum Samples

Initially present	(Cortisol µg/dL) Added	Recovered	Recovery, %
7.7	9.1	9.8	108
11.9	9.1	8.2	90
13.4	9.1	8.1	89
5.2	9.1	10.0	110
7.8	9.1	10.1	111
8.5	9.1	9.4	103
6.6	9.1	9.5	104
17.3	9.1	8.4	92
8.5	9.1	9.0	99
17.6	9.1	7.9	87
25.3	18.2	16.3	90
11.6	18.2	18.6	102
1.22	18.2	17.5	96
4.33	18.2	16.1	88
21.5	18.2	21.7	119
8.4	18.2	20.4	112
14.6	18.2	14.2	78
25.2	18.2	19.9	109
			99.3 ± 11.1%

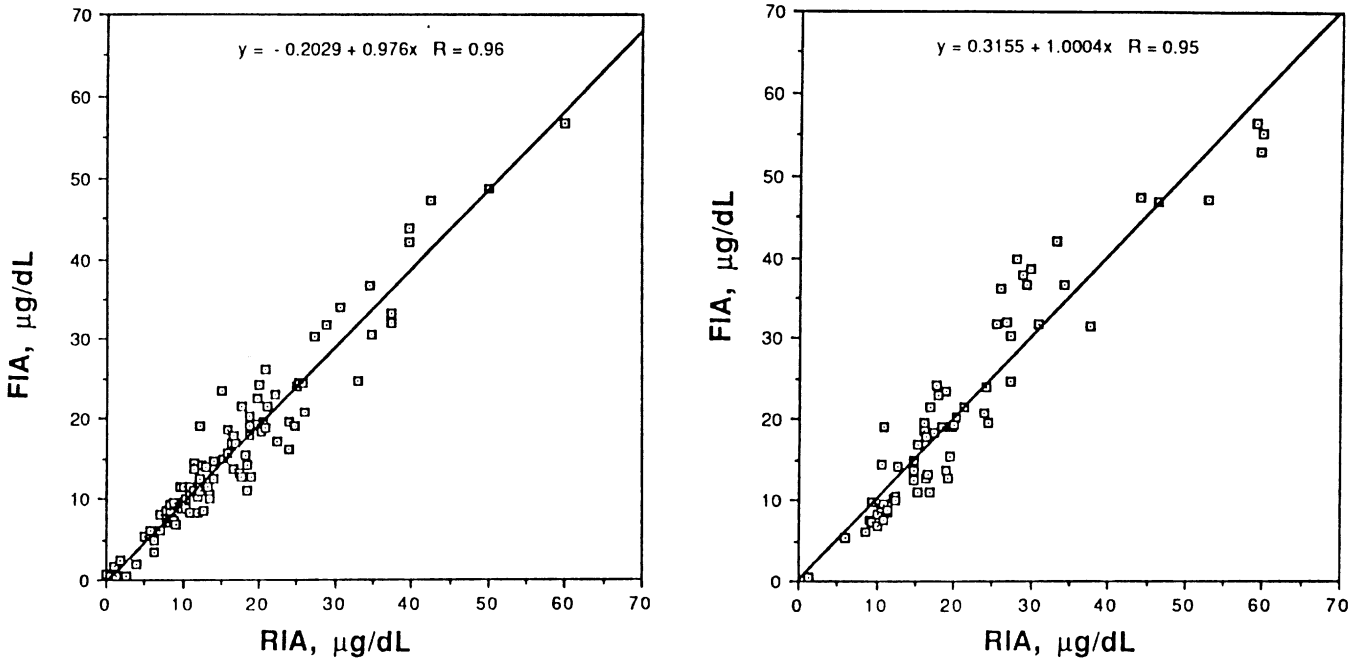

Figure 2—Comparison of the proposed method with two widely used radioimmunoassay procedures. Left panel DPC RIA; right panel Dade RIA.

Discussion

The CFI 615 Immunoanalyzer is the first time-resolved fluorometer designed for solid-phase measurements. The optical diagram of the instrument is given in (2). Briefly, the CFI 615 uses a pulsed nitrogen laser as the excitation source, at 337.1 nm. The pulse duration of the laser beam is 3–4 ns. The repetition rate is 20 pulses/s. Each measuring cycle lasts 50 ms. The sequence of events during one measurement cycle in the time-resolved mode is as follows: excitation beam duration 3–5 ns; delay time during which the photomultiplier is inactive 200 µs; measurement window 200–600 µs; recovery time until the next pulse 49.4 ms. The measurement time per microtitration well is 1 s, so that 20 readings can be taken at maximum. Sixteen readings are averaged and used for analytical purposes. The other four readings are aborted because they coincide with the movement of the plate during readings. During the 200 µs delay time, any short-lived background fluorescence decays and thus does not contribute to the final reading. The laser beam stability is monitored with a vacuum photodiode, and raw fluorescence readings are corrected before being used for analytical purposes. The instrument is equipped with two compact diskette drives and appropriate software for automatic data reduction, self-diagnostic tests, quality control features, and so on. Operation is facilitated through function keys and menu selections appearing on the instrument's screen. A commercially available time-resolved fluorometer (8) and some others that have been reported recently (9,10) are suitable for measurements only in solution.

The performance characteristics of the cortisol assay are similar to those of other immunoassay techniques. The detection system used is based on labeled streptavidin and biotinylated antibodies. The same detection system can be used for any other competitive or noncompetitive immunoassay. Successful examples include the time-resolved immunofluorometric assay of alpha-fetoprotein (3) and human choriogonadotropin (4). The advantages of the biotin-streptavidin system as applied to immunoassays and especially in the CyberFluor analytical system have been summarised elsewhere (2).

The competitive immunoassay procedure employed uses the immobilized antigen instead of the widely used immobilized antibody approach. The immobilized antigen approach is advantageous in many aspects. Antigen-protein conjugates can be easily prepared, and biotinylated antibodies can be used with a universal detection system based on avidin or streptavidin, such as the one proposed here. Synthesis of antigen-enzyme or antigen-fluor conjugates is unnecessary with the immobilized antigen approach. This strategy, however, works best if the antibody used is monoclonal or highly purified polyclonal. With conventional γ-globulin fractions the background is considerably higher because of the labeling (in this case with biotin) of any irrelevant antibodies present. The cortisol-thyroglobulin conjugate used was selected from the cortisol-conjugates with BSA and ovalbumin because of better reproducibility in the coating and higher B_0 values. With the procedure given for labeling streptavidin with BCPDA, conjugates with a BCPDA:streptavidin ratio of about 15 are obtained. The ratio can be easily established by absorbance measurements at 325 nm, where only BCPDA absorbs. This ratio is optimum in terms of assay sen-

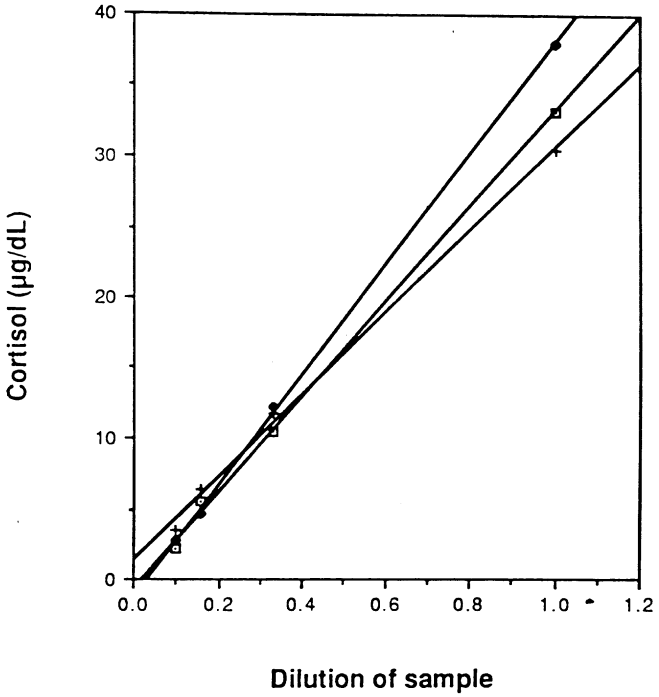

Figure 3—Dilution linearity of the proposed procedure. The regression equations are: $y = -1.36 + 39.3x$, $r = 0.9996$ (●); $y = -0.73 + 33.9x$, $r = 0.9995$ (□); $y = 1.28 + 29.4x$, $r = 0.9987$ (×).

sitivity and streptavidin binding activity. At higher ratios the binding activity of streptavidin decreases significantly.

The cross-reactivity of the monoclonal antibody used is high for prednisolone (this is a limitation of all the antibodies used for cortisol immunoassays), prednisone, cortisone and corticosterone, and to a lesser extent to 11-deoxycortisol and a number of other steroids. The assay is not suitable for measuring cortisol in the serum of patients receiving the cross-reacting steroids as drugs. In terms of cross-reactivity, the monoclonal antibody used is inferior if compared to the best polyclonal antibodies reported for cortisol. However, cortisol is the major steroid in serum and only rarely physiologically present cross-reacting steroids may build-up at concentrations capable of producing a positive error. On the other hand, this monoclonal antibody has a high affinity constant (1×10^9 M^{-1}), is suitable for use in competitive immunoassays with the immobilised antigen approach, and has all the well-known advantages of monoclonal antibodies (purity, continuous availability, low cost, etc.).

The sensitivity of the chemical system used is not exploited to its full capacity with the cortisol assay. Much higher assay sensitivities can be achieved. However, in the cortisol assay, the major concern was to use a sample volume that can be easily and reproducibly pipetted and at the same time maintaining a wide assay range to avoid frequent dilution and re-run of high cortisol samples.

Cortisol is bound to serum proteins and for a successful competitive immunoassay it has to be released. We have tested a number of frequently used agents for this purpose, *e.g.*, 8-anilino-1-naphthalene sulfonic acid, salicylate, and merthiolate. We have also used trichloroacetic acid, a new reagent proposed recently by Eskola *et al.*(11). The latter reagent at a final concentration of 0.4 M gave the most favourable results and it was thus used for the assay.

An important advantage of time-resolved fluorometric assays is the speed of measuring the final product in the microtitration wells. A 96-well plate can be read in about 2 min. Data reduction and printout of a report including a calibration curve takes an additional 3 min on the CyberFluor 615™ Immunoanalyzer. A unique feature of the present system is that the dry wells can either be stored and read at a convenient time during the same working day or at a later opportunity. The fluorescence of the dry wells was found to be very stable upon storage (six months or more). This feature is attractive and is not found in competing technologies of liquid-phase time-resolved fluorometry (12,13), enhanced luminescence, and enzyme immunoassay. Additionally, the handling of dry wells during measurement is more convenient to the operator in comparison to wells full of liquids.

The replacement of radionuclides with alternative labels is highly desirable due to the many and well-known associated disadvantages of radioactivity. Time-resolved fluorescence is a promising alternative methodology in the field of non-isotopic immunoassay. The relative merits and disadvantages of using either Eu^{3+} (12,13) or Eu^{3+} chelates (like BCPDA) as labels have been discussed elsewhere (2–4). A notable difference between the two approaches is that with Eu^{3+} as label, the assays are vulnerable to exogenous Eu^{3+} contamination.

In conclusion, we present the first competitive heterogeneous immunoassay of a hapten (cortisol) with a new chemical system that we have introduced recently. The integrated system (instrument + chemistry) is currently being evaluated for the development of sensitive immunoassays for other analytes of clinical importance.

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