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Laser-Excited Time-Resolved Solid-Phase Fluoroimmunoassays with the New Europium Chelate 4,7-Bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic Acid as Label

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We have prepared biologically active highly fluorescent monoclonal and polyclonal antibodies suitable for time-resolved immunoassays. We have first labeled bovine serum albumin (BSA) with the new europium chelate 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA). We have incorporated maleimide groups on the antibody molecules by using the heterobifunctional reagent sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate. We then reduced the labeled BSA with dithiothreitol and conjugated the labeled BSA with the derivatized antibody. We have tested the labeled antibodies on a model heterogenous competition fluoroimmunoassay for serum cortisol and obtained satisfactory results. The amount of the bound antibody, which was inversly related to the amount of cortisol in the sample, was quantitated by measuring, in a time-resolved mode, the fluorescence of the complex antibody–BSA–BCPDA–Eu^{3+} bound to cortisol immobilized on the solid phase after excitation with a nitrogen laser beam and monitoring delayed fluorescence at 615 nm.

Radioimmunoassays (RIA) and immunoradiometric assays (IRMA) are among the most sensitive and specific analytical techniques available today (*1*). Their exceptional performance characteristics arise from the use of antibodies as analytical reagents (specificity) and radioactive labels as the detection system (sensitivity). Immunoassay in general, is a universal analytical technique and has been used successfully for the assay of polypeptide, steroid, and thyroid hormones, drugs, metabolites, tumor markers, antigens, and antibodies of infectious agents, etc. The detection system used to monitor the immunological reaction need not be restricted to radioactive labeling. Despite their advantages which facilitated their establishment during the last 25 years (very high sensitivity of detection, invulnerability to environmental interference, accuracy and precision in measurement of signal, no background signal) radioactive labels have a number of serious disadvantages (potential health hazard, special licensing for their use, special disposal, unstable reagents, difficulty in automation) which prompted research for their replacement. During the last few years, in the field of clinical chemistry, a number of alternative labels have been explored. The most successful alternative labels currently used in both research and commercial applications are enzymes (*2, 3*), luminescent labels (*4, 5*), and fluorescent labels (*6, 7*).

Fluorescein, the most widely used conventional fluorescent label was very successful in applications where extreme sensitivity is not required, i.e. in therapeutic drug monitoring assays. For highly sensitive immunoassays, fluorescein is not suitable because of the following limitations. It has only ~28 nm Stokes shift, the emission spectrum overlaps extensively with the emission spectrum of serum autofluorescence, and the molecule quenches the fluorescence of adjacent fluorescein molecules so that highly labeled reagents are not very useful. Recently, there is increasing interest in the use of rare earth metal complexes in devising new immunoassay systems (*8*). The fluorescence of Eu^{3+} complexes with certain organic ligands has some interesting properties: (a) there is a very large Stokes shift of the order of ~290 nm. This is due to the fact that the excitation radiation is absorbed by the organic ligand and the energy is then transferred to the rare earth metal ion which emits its characteristic radiation at a very long wavelength (~615 nm for Eu^{3+}) (*9*). (b) The emission spectrum is very narrow (~10 nm for Eu^{3+} at 50% emission bandwidth). These two characteristics allow for very easy and specific isolation of the emitted light by using an interference filter at ~615 nm. Additionally, the fluorescent lifetime of these complexes is very long (between 10 and 1000 μs) as compared to that of conventional fluorescent molecules (e.g. 49.5 ns for fluorescein). This property allows for fluorescence to be measured with a "time-resolved" technique as follows: After the molecule is excited with a short pulse of light, the emitted fluorescence is measured in a properly selected time window. Measurement starts after an initial delay time during which any short-lived fluorescence has decayed. When all the features of the fluorescence of europium complexes are taken into account (large Stokes shift, narrow emission band, long-lived fluorescence) in devising the instrument and chemical system for an immunoassay, it is possible to exclude practically all sources of background fluorescence and thus achieve very high sensitivity of specific signal detection. The only limiting background fluorescence measured, will be due to nonspecific binding of the labeled reagents used.

There are two general approaches in devising analytical immunoassay techniques with europium complexes as the detection system. The first approach is to introduce Eu^{3+} into the immunoreactants to create the label and then excess chelate to form the fluorescent complex (*10*). This approach has been used successfully but suffers from some limitations; the most important being the vulnerability to Eu^{3+} contamination. The second approach is to introduce the chelator into the immunoreactants and use excess Eu^{3+} to form the fluorescent complex (*11, 12*). This approach, although tried in the past (*13*), was not successful because of the lack of appropriate Eu^{3+} chelators. Such chelators should combine the following characteristics: a suitable reactive group for incorporation into proteins; a Eu^{3+} chelating site with a high stability constant for the complex; a suitable excitation wavelength with high extinction coefficient; and an effective energy transfer mechanism to Eu^{3+}. A molecule which fulfills these criteria has been synthesized recently (*11*). Its appli-

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cability for immunoassays is discussed in detail elsewhere and practical examples are given (*14*).

In this report, we describe a method for labeling antibodies with this novel Eu^{3+} chelator. We have concentrated on two major considerations; how to introduce amplification to the system (high molar ratio of chelator/antibody) yet keep the antibody modification as low as possible so as to preserve the biological activity. We have selected to first exhaustively label a carrier protein, bovine serum albumin (BSA), with the chelator and then cross-link the labeled protein to the antibody molecule with a bifunctional reagent. We have demonstrated that high yields of active labeled antibodies can be achieved by using polyclonal and monoclonal anti-cortisol antibodies as a model system. These antibodies have been used successfully for devising a heterogeneous competition assay for serum cortisol, with ovalbumin–cortisol conjugate immobilized in microtitration wells as the solid phase.

EXPERIMENTAL SECTION

Materials. Bovine serum albumin (BSA, RIA grade), ovalbumin, hydrocortisone 21-hemisuccinate, and hydrocortisone 21-hemisuccinate BSA conjugate (cortisol-21-BSA) were purchased from Sigma Chemical Co., St. Louis, MO. Tri-*n*-butylamine was purchased from Aldrich Chemical Co., Milwaukee, WI, isobutyl chloroformate was from Chemical Dynamics Corp., South Plainfield, NJ, sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) was from Pierce Chemical Co., Rockford, IL, *N*-ethyl maleimide was from Eastman Kodak Co., Rochester, NY, and 2,2′-azinobis[3-ethylbenzthiazolinesulfonate (6)] (ABTS) was from Boehringer Mannheim (Canada), Dorval, PQ. Gelatin (EIA purity) was obtained from Bio-Rad Laboratories (Canada), Ltd., Mississauga ON, and Sephadex G25 medium mesh from Pharmacia (Canada), Ltd. A radioimmunoassay (RIA) kit for cortisol, Coat-A-Count, was purchased from Diagnostic Products Corp., Los Angeles, CA. Europium chloride was purchased from Aldrich and a stock solution of 1 mM was prepared in 0.01 M HCl. 4,7-Bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (Eurofluor S, BCPDA) was synthesized according to the procedure of Evangelista et al. (*11*). All other chemicals were reagent grade. Monoclonal antibody to cortisol was purchased from Medix Biotech, Inc., Foster City, CA, and polyclonal rabbit anti-cortisol (against cortisol-21-BSA) was purchased from Western Chemical Research Corp., Ft. Collins, CO. Protein concentration was carried out by centrifugation using Centricon 30 microconcentrators from Amicon Canada, Ltd., Oakville ON. The anti-rabbit IgG and anti-mouse IgG coupled to horseradish peroxidase were purchased from Tago Immunologicals, Burlingame, CA.

Enzyme immunoassay (EIA) microtitration plates were obtained from Flow Laboratories, Inc., McLean, VA, and read on a EL309 microplate reader, Bio-Tek Instruments, Inc., Winooski, VT. Fluoroimmunoassay (FIA) microtitration plates, Microfluor W, white opaque 96-well plates were purchased from Dynatech Laboratories, Inc., Alexandria, VA, and read on the CyberFluor 615 fluorometer.

High-performance liquid chromatography (HPLC) was carried out with a BioSil TSK 400 size exclusion column from Bio-Rad Laboratories on a Model 600 gradient system equipped with a 490 variable wavelength detector (Waters, a division of Millipore (Canada), Ltd., Mississauga, ON).

Instrumentation. A specially designed instrument has been used for solid-phase time-resolved fluorescence measurements (CyberFluor Model 615 Immunoanalyzer). The instrument is essentially a gated fluorometer and consists of a nitrogen laser as the exciting source, a novel optical system, and a direct current technique for the quantitation of fluorescent light intensity. Measurements can be carried out in microtitration plates or strips with a measuring time of 1 s per well. Specially designed software allows for automated data reduction by spline smoothing techniques. A brief description of the instrument is given in ref 14.

Methods. *Preparation of Cortisol–Ovalbumin Conjugate.* Cortisol ovalbumin was prepared by the mixed anhydride method (*15*). Fifty milligrams (0.1 mmol) of hydrocortisone 21-hemisuccinate was dissolved in 10 mL of dioxane and 0.1 mL of tri-*n*-butylamine added. The solution was cooled to 10 °C, 0.02 mL of isobutyl chloroformate added, and the mixture stirred for 30 min. Then, 500 mg of ovalbumin (0.01 mmol) dissolved in 10 mL of water adjusted to pH 9 with NaOH was added to the reaction mixture and the solution was stirred 24 h at 4 °C. After the reaction was completed, a precipitate was present. The mixture was dialyzed for 36 h against water and the precipitate was removed by centrifugation. Urea was added to the supernatant to achieve a concentration of 6 M and this solution was again dialyzed exhaustively against water. The protein concentration was determined by the Bio-Rad protein assay.

Conjugation of BCPDA to BSA. Two milliliters of 0.5 M sodium carbonate buffer of pH 9.1 was added to 250 mg of BSA dissolved in 2 mL of water. One hundred milligrams of BCPDA (50 × molar excess) dissolved in 400 μL of dimethylformamide (DMF) was added in five portions over a 5-min period. Unreacted BCPDA was removed by exhaustive dialysis against 0.1 M $NaHCO_3$ and the labeled BSA (BSA–BCPDA) stored at 4 °C.

Reduction of BSA–BCPDA. Reduction was carried out just prior to conjugation with antibody. To BSA–BCPDA (30 mg/mL in 0.1 M $NaHCO_3$) solid urea, dithiothreitol (DTT), and Tris base were added to achieve a concentrations of 6 M, 50 mM, and 0.1 M, respectively. The pH measured was 9. The mixture was incubated for 1 h at 37 °C.

Conjugation of Antibody and Reduced BSA–BCPDA. Conjugation with sulfo-SMCC was carried out by a modification of the method of Yoshitake et al. (*16*). 0.5 mL of anti-cortisol antibody solution (0.5 mL, 1 mg/mL) was dialyzed overnight against 0.1 M sodium phosphate buffer at pH 7.0 A 14.5-μL portion of a 5 mg/mL solution of sulfo-SMCC dissolved in the same buffer was added and the solution shaken for 1 h at room temperature. Unreacted sulfo-SMCC was removed by desalting on a 25-mL column of Sephadex G 25 using 0.1 M sodium phosphate of pH 6.2 containing 5 mM ethylenediaminetetraacetate (EDTA) as the elution buffer. The fractions containing protein were combined and concentrated to 0.1–0.2 mL by centrifugation in a Centricon 30 device. A 0.35-mL sample of reduced BSA–BCPDA containing 9 mg of protein was also desalted to remove excess reducing reagent on a 25-mL Sephadex G25 column in the same phosphate buffer of pH 6.2, containing 5 mM EDTA, to retard disulfide formation (*17*). The protein containing fractions were combined and concentrated by centrifugation in a Centricon 30 device, to 0.6–0.8 mL. It is important to complete the reactions and the separation and concentration steps as quickly as possible to prevent hydrolysis of the maleimide group in the antibody and re-formation of the disulfide bonds in the reduced BSA. The derivatized antibody and reduced BSA were combined and incubated 20 h at 4 °C (BSA to IgG ratio is 30–40-fold). Prior to purification of the IgG-BSA conjugate, excess SH groups on the reduced BSA molecule (36 SH groups exist per reduced BSA molecule (*17*) were blocked by addition of a 2-fold excess of *N*-ethylmaleimide in DMF and incubation for 1 h at room temperature.

Isolation of BSA-Conjugated Antibody. The conjugated and unconjugated antibody and unconjugated labeled BSA were separated by size exclusion HPLC on a BioSil TSK 400 column (300 × 7.5 mm) eluted at 1 mL/min with 50 mM Na_2SO_4 and 20 mM sodium phosphate pH 6.8 and fractions of 0.5 mL were collected automatically. Three or four injections of 250 μL each were required per preparation to complete the isolation. Fractions were analyzed for total fluorescence, fluorescent antibody, and total antibody concentration, as described below.

Total fluorescence was measured by addition of 200 μL of 10^{-5} M Eu^{3+} solution in 50 mM Tris buffered saline pH 7.8 (TBS) to 5 μL of each fraction and determining the fluorescence of the solution after 5 min, on an ARCUS gated fluorometer (LKB Wallac, Turku, Finland).

To measure the BSA–conjugated antibody concentration, 100-μL portions of fractions diluted 1/20 and 1/200 in 1% BSA in TBS were added to the wells of coated Microfluor W plates. Coating was done overnight at 4 °C with 100 μL per well of a 5 μg/mL solution of cortisol–BSA conjugate in 0.1 M sodium bicarbonate and afterward the plates were blocked for 1 h at room temperature with 1% BSA in the same buffer. The plates were incubated for 1 h at 37 °C and then washed three to four times with water. One hundred microliters of 1×10^{-5} M Eu^{3+} solution

in TBS was then added. After 5 min, the plates were washed once with water and dried in a stream of cool air. Surface fluorescence of the antibody–BSA–BCPDA–Eu^{3+} conjugate which was bound to the immobilized cortisol–BSA was measured in a CyberFluor 615 fluorometer.

The total antibody activity (conjugated to BSA and unconjugated) was measured by using a second antibody (anti-rabbit IgG or anti-mouse IgG for polyclonal or monoclonal anticortisol, respectively) coupled to horseradish peroxidase (HRP) in an enzyme-linked immunosorbent assay (ELISA). EIA plates were coated with cortisol–BSA conjugate and blocked as above. HPLC fractions were diluted, added to the EIA plate, and incubated at 37 °C as above. After the plates were washed with water, 100 µL of horseradish peroxidase labeled goat anti-rabbit or anti-mouse IgG diluted 1/500 with 50 mM sodium phosphate pH 8.5 containing 1% NaCl and 1% BSA was added and the plates were incubated a further hour. The plates were washed with water and the enzymic activity of the bound anti-IgG–HRP bound to antibody which in turn was bound to immobilized cortisol–BSA was measured by adding 100 µL of substrate (1 mg/mL of ABTS in 50 mM Na_2HPO_4, 25 mM citric acid, 0.03% H_2O_2). The optical density was determined on a microplate reader after 5–10 min.

Cortisol Assays. We used a solid-phase competition assay with immobilized antigen to measure cortisol. Microfluor W plates were coated overnight at 4 °C with 100 µL of 2 µg/mL cortisol–ovalbumin conjugate in 0.1 M $NaHCO_3$. The plates were rinsed once with water and blocked for 1 h at room temperature with 0.1% gelatin, 0.1% Tween 20, and 50 mM phosphate buffer of pH 7.4 and stored in the same solution at 4 °C.

Before the assay, a plate was washed twice with 0.05% Tween 20 in saline (Tween/NaCl) and twice with water. Antibody was diluted to 1/400 with respect to the starting material (1 mg/mL) in TBS containing 0.3 M trichloroacetate (TCA), 1% BSA, and 150 µg/mL BSA–BCPDA to prevent fluor–serum interactions. Ten microliters of serum was pipetted into the wells and 100 µL of antibody solution added. The plate was briefly shaken and incubated 1 h at 37 °C. The plate was washed three times with Tween/NaCl and twice with water, then 100 µL of 1×10^{-5} M Eu^{3+} in TBS was added. After 5 min, the plate was rinsed once with deionized water and dried with a stream of cool air and surface fluorescence was determined on a CyberFluor 615 fluorometer.

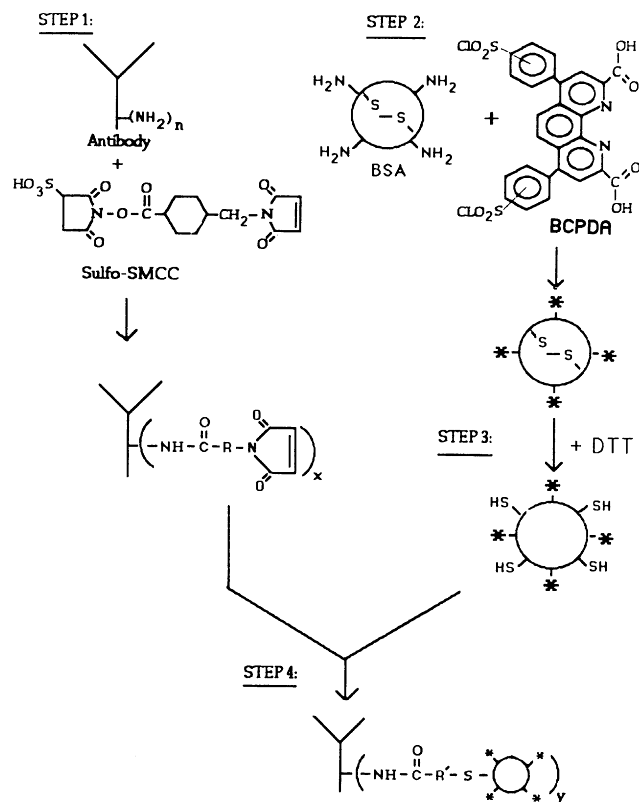

Figure 1. Procedure for the preparation of antibody–bovine serum albumin (BSA)–4,7-bis(chlorosulfophenyl)-1,10 phenanthroline-2,9 dicarboxylic acid (BCPDA) conjugates. In step 1 antibody is reacted with sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) to introduce maleimide groups. In step 2 BSA is coupled to BCPDA. In step 3 labeled BSA is reduced by dithiothreitol (DTT) to create free SH groups. In step 4 the derivatized antibody is coupled to reduced BSA. Numbers *n*, *x*, and *y* represent the number of total amino groups, derivatized amino groups, and conjugated amino groups, respectively.

RESULTS AND DISCUSSION

Conjugation of Antibodies. The coupling scheme which we used to conjugate BSA–BCPDA to antibody is illustrated in Figure 1. The antibody, which has no free sulfhydryl groups, is reacted with a bifunctional coupling reagent containing an amino reactive *N*-hydroxysuccinimide moiety at one end and a sulfhydryl reactive maleimide group at the other end. The sulfonic acid group renders the reagent water soluble. The reagent is reacted first with the antibody to introduce maleimide groups (step 1). The BSA which has been exhaustively reacted with BCPDA (step 2) is reduced with DTT to create free sulfhydryl groups from intramolecular disulfide bonds in the native molecule (step 3). The exposed SH groups are then free to react with the maleimides introduced into the antibody (step 4).

Since it is possible to get inactivation of antibody at high concentrations of coupling reagent, we studied the effect of the concentration of coupling reagent in step 1 on the activity of the derivatized antibody preparation using both a polyclonal and a monoclonal anti-cortisol antibody. Antibodies were reacted for 1 h at varying molar excess of sulfo-SMCC in step 1. The titer of the modified antibody was measured by ELISA, using an HRP conjugated anti-IgG, in microtitration plates, as described in Methods (isolation of conjugated antibody). As shown in Figure 2 the monoclonal antibody preparation was highly active even after derivatizing with 500-fold excess of sulfo-SMCC; the polyclonal antibody inactivation was evident even at 25-fold molar excess of sulfo-SMCC (Figure 2B). The monoclonal was therefore treated with a 50-fold molar excess of sulfo-SMCC while the polyclonal was treated with a 20-fold excess, in step 1. Because of the possibility of cross-linking networks in step 4 due to the reaction of one BSA molecule bearing many SH groups with multiple molecules of derivatized antibody and also, of one derivatized antibody molecule with multiple molecules of reduced BSA, the reduced BSA/derivatized antibody molar ratio was selected to be very high (>25-fold) so as to promote formation of $IgG(BSA)_n$ rather than $BSA(IgG)_n$.

After conjugation, the conjugated and unconjugated antibody were separated by gel filtration on HPLC. The fractions were monitored for optical density, total fluorescence, fluorescent antibody binding to cortisol–BSA coated plate, and total antibody binding to cortisol–BSA coated plates, using a second peroxidase conjugated anti-IgG antibody in an ELISA assay as described in Methods. The results of a typical monoclonal preparation are shown in Figure 3. There is a continuous spectrum of fluorescent antibodies present with different molecular weights, with a large fraction in the void volume. There are three peaks of total antibody activity as measured by the ELISA technique (Figure 3B), the void volume (peak A), an included peak also corresponding to coupled antibody (peak B), and an included peak corresponding to uncoupled antibody (peak C). Only peak A and the first fractions of peak B were combined for use in the assays so as to ensure that essentially all uncoupled antibody is excluded. Assuming that the binding of the second antibody HRP–conjugated anti-IgG is similar for all the fractions of anti-cortisol, irrespective of the degree of conjugation to BSA, 30–50% of the antibody is recovered in the conjugated antibody fractions combined and used for the immunoassay.

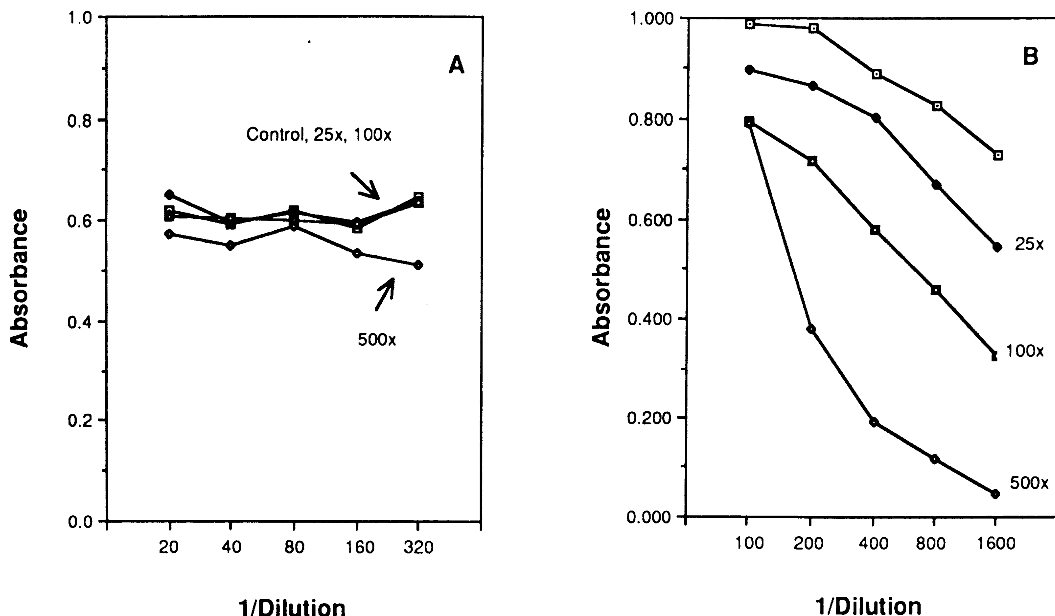

Figure 2. Titer of monoclonal (left) and polyclonal (right) antibodies after modification with sulfo-SMCC. Antibodies were modified with various molar excess of sulfo-SMCC as described under procedures. The antibody titer was then determined by ELISA as discussed under isolation of conjugated antibody.

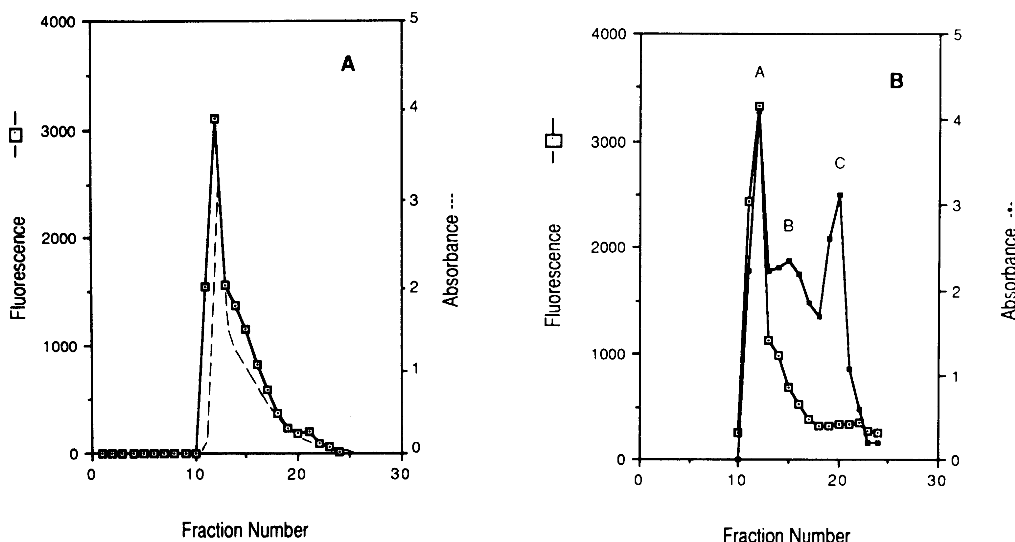

Figure 3. Separation of antibody-BSA-BCPDA conjugate on size exclusion HPLC: (A) (- - -) absorbance, (□) total fluorescence of fractions measured by diluting 5 μL of each fraction in 200 μL of 10^{-5} M Eu^{3+} in TBS; (B) (●) total (conjugated and unconjugated) antibody concentration as measured by HRP-conjugated anti-mouse IgG in an ELISA assay, (□) conjugated fluorescent antibody binding to plates coated with ovalbumin-cortisol. For more details see text.

Assay Optimization. An outline of the cortisol competition assay is shown in Figure 4. The calibration curves for the cortisol assays were plotted in one of two ways: (a) the fluorescent readings for the standards were plotted vs the log of the cortisol concentration or (b) the ratios of the fluorescence of the standards, B, to the fluorescence of the zero standard, B_0, expressed as a percentage ($B/B_0 \times 100\%$) were plotted vs the log of the cortisol concentration.

Variation in the amount of coating in the microtitration wells from 50 to 500 ng of cortisol–ovalbumin conjugate had little effect on the overall shape of the calibration curve when this is plotted as (B/B_0), but the total antibody bound to the plate (proportional to B_0) reached its maximum at a coating of 200 ng/well. We found that calibration curves with the conjugated polyclonal antibody became flat with no further change in ($B/B_0 \times 100$) when this value reached 30%, regardless of increasing cortisol concentration, whereas the curves with conjugated monoclonal continued to drop until

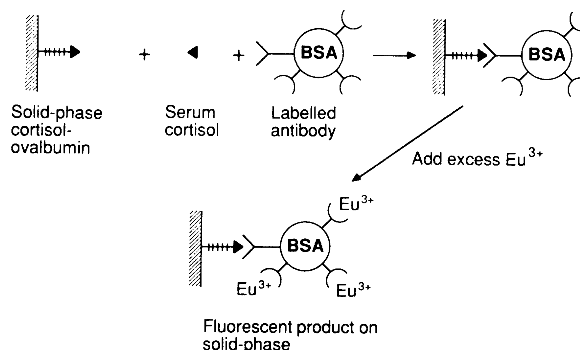

Figure 4. Principle of the competition assay of serum cortisol. Washing steps are not shown; more details are given under procedures. The figure has no quantitative meaning. The bond between BCPDA and BSA is covalent. Fluorescence is measured directly on the solid phase. BCPDA is represented schematically (⊃–). BSA represents bovine serum albumin.

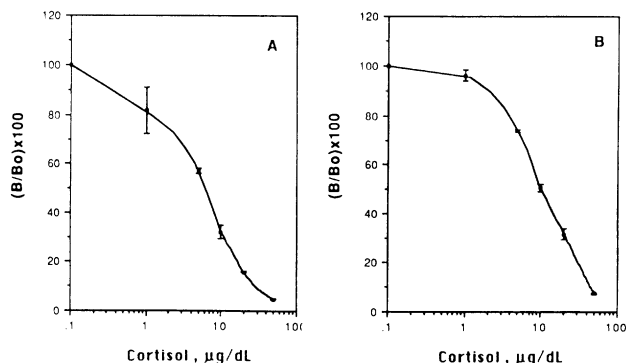

Figure 5. Standard curves for the cortisol assay in serum. Each point is the mean (± standard deviation) of three determinations: (A) 20-μL sample volume; (B) 10-μL sample volume.

Table I. Within-Run Precision of the Cortisol Assay

sample	no. of determinations	mean value, μg/dL	std dev, μg/dL	% CV[a]
1	9	5.6	0.6	10.2
2	10	14.8	1.0	7.0
3	10	24.8	1.3	5.3
4	9	53.8	0.7	1.4

[a] Percent coefficient of variation.

a value of ($B/B_0 \times 100$) close to zero was obtained. We thus continued experimentation exclusively with the monoclonal antibody.

Since >80% of the cortisol in serum is bound to transport proteins (*18*), a suitable dissociation reagent is required to release the cortisol before measurement by immunoassay. By use of the monoclonal antibody, two reagents were investigated: 8-anilino-1-naphthalenesulfonic acid, ANS, at a concentration of 5% in the final assay mixture and trichloroacetate, TCA (*19*), at a concentration of 0.3 M. TCA gave better results than ANS and thus it was preferred in the final assay design.

We tested the volume of serum required in order to obtain sensitive calibration curves. This parameter affected the shape of the calibration curve dramatically. The normal range of cortisol in human serum is 5–30 μg/dL (*19*). Using 20 μL of serum, we found that the curves were too shallow at concentrations greater than 20 μg/dL. A better calibration curve is obtained with 10 μL of sample volume. With 10 μL of serum (Figure 5B), good sensitivity was achieved in the whole range of clinically important values (1–50 μg/dL).

Assay Performance. A typical calibration curve is shown in Figure 5B. The steepest part of the curve is in the normal range where maximum accuracy and precision are usually needed.

The within-run precision for four patient samples is shown in Table I. This kind of precision (coefficient of variation of 2–10%) is typical of well-established cortisol assays currently available.

To test the linearity of the method, we have diluted four patient samples with the zero standard and reassayed them. We found a linear relationship between the measured concentration and the dilution, indicating that our assay is free from any serum matrix effects.

The recovery of added cortisol was measured in five different samples. The recovery of cortisol is shown in Table II and varied from 91% to 115%, with a mean of 102%.

We measured 27 patient samples by a commercial RIA kit (Diagnostic Products Corp.) as well as with the present method with conjugated fluorescent monoclonal antibody. The samples were selected to cover the whole range of the assay from

Table II. Recovery of Added Cortisol in Serum

sample	cortisol, μg/dL initially present	added	found	% recovery
1	1.3	3.0	3.5	115
1	1.3	11.0	12.2	111
2	6.6	9.3	8.4	91
2	6.6	15.3	14.9	97
3	10.9	12.7	12.0	95
3	10.9	18.7	18.8	101
4	15.2	22.2	22.9	103
5	18.2	24.5	26.5	108
6	35.0	38.0	36.9	97

1 to 50 μg/dL and included clear, cloudy, lipemic, and haemolytic specimens. The correlation between the two assays was found to be good. The coefficient of correlation is 0.98 with a slope of 1.007 and an intercept of 0.51 μg/dL.

Time-resolved fluoroimmunoassays perform better than conventional fluoroimmunoassays in biological fluids because of effective background rejection. Currently, the most appropriate fluorescent probes for time-resolved immunoassays appear to be the Eu^{3+} complexes. In addition to their long fluorescence lifetimes, these complexes have the advantages of large Stokes shifts and sharp emission bands. When Eu^{3+} is introduced into the immunoreactants as label, the assays are vulnerable to Eu^{3+} contamination from the skin, dust, etc. Therefore, during the performance of such assays, great care is needed in order to exclude the contamination sources. With the synthetic Eu^{3+} chelates as labels, excess Eu^{3+} is used and contamination problems are virtually eliminated.

The new Eu^{3+} chelate 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid, BCPDA, can be introduced covalently into the immunoreactants, by means of reaction of the sulfonyl chloride group, primarily with available amino groups of the antibody or antigen used. This reagent has two available SO_2Cl groups; thus, there is a possibility of polymer formation because proteins have also more than one amino group available. We have investigated this possibility by carrying out labeling experiments with BSA and separating the products by using HPLC with size exclusion columns (data not shown). We found that working with BCPDA in excess over BSA, the major product was an adduct of the form BSA(BCPDA)$_n$. The value for n is between 1 and 40 depending on the molar excess used. It was assessed by measuring the absorbance of BCPDA at 325 μm; $\mathcal{E} = 1.52 \times 10^4$ M^{-1} cm^{-1}. Protein–protein linkage, as assessed by the size of the peak at the void volume, was minimal.

Antibodies are highly reactive biological molecules. Upon extensive derivatization, they may lose their ability to bind to their respective antigens. For that reason, it is preferable to label antibodies with the minimum amount of the label used. On the other hand, extensive labeling is desirable because of the increased sensitivity in detection. Our approach for labeling antibodies combines the low degree of antibody modification with the high degree of labeling, by using BSA as a label carrier. The carrier protein is exhaustively labeled in one step and then cross-linked under mild conditions with the polyclonal or monoclonal antibody by using a bifunctional reagent.

The bifunctional reagent used, sulfo-SMCC, is now considered one of the state-of-the-art heterobifunctional cross-linkers. It is superior to the classical carbodiimides and glutaraldehyde reagents which have been used for creating protein–protein conjugates. Because it is heterobifunctional, antibody–antibody conjugates during the first step of the reaction (Figure 1) are not formed. However, it is still very likely to obtain a heterogenous population of conjugates as the final product since one antibody molecule, carrying many

maleimide groups, can react with one or more reduced labeled BSA molecules. It is also possible to obtain, for the same reason, conjugates consisting of one labeled BSA molecule and many antibody molecules. The latter possibility is minimized by adding the reduced labeled BSA reagent in great excess over the derivatized antibody, during the final conjugation step (Figure 1, step 4).

The labeled monoclonal antibodies performed better than the labeled polyclonal antibodies in the model cortisol immunoassay. This was expected because the polyclonal antibody preparation contains, in addition to the specific cortisol antibodies, antibodies to the carrier protein and the hemisuccinate bridge of the immunogen, and other irrelevant antibodies as well. During the labeling procedure, the whole population of antibodies is labeled and this leads to increased nonspecific binding during the immunoassay. Additionally, there is binding of the carrier protein antibodies and of the bridge antibodies to the solid-phase which cannot be prevented by cortisol. This leads to calibration curves which appear flat (no change in the value of $(B/B_0) \times 100$ upon increasing the cortisol concentration of the standards) after a certain cortisol concentration. These effects were minimal with the labeled monoclonal antibody preparations.

We have used an ELISA technique to monitor the distribution of both labeled and unlabeled antibody molecules during the HPLC gel filtration separation step. A solid-phase fluorescence technique was also used to monitor the distribution of only the labeled antibody population. Both techniques showed that the labeled antibody preparation is heterogeneous, with most of the activity eluted in the void volume. This heterogeneity is not a problem however, and a successful assay could be devised by mixing the void volume fraction and a limited number of the following fractions which are free from any unlabeled antibody molecules. The final mixture of labeled antibodies could be used in a dilution of about 1:400 (with respect to the initial starting material) which is typical for nonisotopic immunoassays.

The labeling procedure we have presented is general. Antibodies labeled with this method can be used for competitive and "sandwich" type immunoassays of any antigen, provided that specific antibodies are available and the sensitivity is adequate. The performance of the competition cortisol assay, presented here as a model, is satisfactory.

In conclusion, we have reported a new detection system for devising time-resolved fluoroimmunoassays, with the novel Eu^{3+} chelate BCPDA as label. A number of different applications with the same detection system will be published in the near future.

Registry No. Cortisol, 50-23-7.

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