Direct Solid-Phase Time-Resolved Immunofluorometric Assay of Cortisol in Serum

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A dissociation-enhanced lanthanide fluoroimmunoassay of serum cortisol based on time-resolved fluorescence is described. The assay is a direct assay, where cortisol immobilized on the wall of a microtiter-strip well competes with cortisol in the sample for the europium-labeled polyclonal antibody. The amount of bound europium-labeled antibody is inversely proportional to the amount of cortisol in the sample. Separation is accomplished by washing the strip well. The assay is carried out in 2 h, at room temperature; it is easy to perform and gives accurate and reliable results. A chaotropic agent, trichloroacetic acid, was very effective in releasing cortisol from binding proteins. This finding will have practical importance in the immunoassay field.

Materials and Methods

Materials

Sephadex G-50, Sepharose 6B, and Sephadex G-25M columns (PD10) were purchased from Pharmacia, Piscataway, NJ 08854. Bovine serum albumin, bovine gammaglobulin, ovalbumin, and cortisol were from Sigma Chemical Co., St. Louis, MO 63178. Sheep anti-cortisol serum, prepared against cortisol-3-(O-carboxymethylxime)-bovine serum albumin (cortisol-3-CMO-BSA) was donated by Pharmacia. The isothiocyanate derivative of europium chelate (8) and cortisol-3-CMO-N-hydroxysuccinimide (11) were synthesized in the Wallac Biochemical Laboratory. Other chemicals used were of analytical grade.

Methods

Purification and labeling of anti-cortisol IgG. The gammaglobulin fraction was concentrated from sheep anti-cortisol serum by precipitation with solid Na2SO4 (180 g/L) at 25 °C. Prepacked columns of Sephadex G-25M were used for desalting and buffer exchange. The total IgG fraction (16.8 mg/mL, 200 µL) in sodium phosphate buffer (50 mmol/L, pH 7.3) was used for labeling. The isothiocyanate derivative of the Eu chelate (8) was added in 400 molar excess (95 µL) and the pH was adjusted to 9.00 by adding 100 µL of 0.5 mol/L Na2CO3 solution. After incubation at 4 °C for 2 h, the labeled IgG was purified from excess label by using a 1-cm (diameter) column filled to a height of 5.5 cm with Sephadex G-50 and with Sepharose 6B for an additional 52 cm. The incorporation gave a ratio of Eu to IgG of 17.7. The labeled IgG could be stored at 40 °C for at least four weeks without any loss of immunoreactivity.

Cortisol-3-CMO-ovalbumin conjugate. This conjugate was prepared essentially as previously described (11). Cortisol-3-CMO-N-hydroxysuccinimide (1.6 mg) in 1.7 mL of dioxane was added to a solution of 100 mg of ovalbumin in 3.5 mL of the sodium phosphate buffer. The molar cortisol-ovalbumin ratio was about 1.3. The reaction was carried out at 4 °C for 20 h. The conjugate was first filtered through a 0.25-µm (pore-size) membrane (Millipore Corp., Bedford, MA 01730) and then purified by passage through Sephadex G-25 columns. The cortisol/ovalbumin ratio, calculated on the basis of the ultraviolet absorption spectra, was about 1.

Coating of polystyrene microtiter strips. The cortisol-3-CMO-ovalbumin conjugate was immobilized by adsorption onto the walls of the wells of polystyrene microtiter strips. Each strip contains 12 wells, each 6.5 (diameter) × 11 mm. The wells were coated at room temperature for 20 h with 100 µL of 0.5 µg/mL conjugate solution in Tris buffer (50 mmol/L, pH 7.4) containing 9 g of NaCl and 500 mg of Na3PO4 per liter (TSA buffer). The wells were then washed, first three times with a solution containing 9 g of NaCl, 500 mg of Na3PO4, and 0.5 g of Tween 20 polyoxyethylene (20) sorbitan monolaurate per liter, then three times with the same solution without Tween 20. Finally, the strips were air-dried and stored in plastic bags until use. The coated strips were ready for use.

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Received March 21, 1985; accepted July 10, 1985.
are very stable. No loss of immunoreactivity was observed on storage for four weeks at 40°C.

Displacement of cortisol from endogenous binding proteins. Before use, the concentrated Eu label was diluted 20-fold with assay buffer (pH 7.75) having the following composition per liter: sodium chloride 9 g, bovine serum albumin 5 g, sodium azide 0.5 g, bovine gamma-globulin 0.5 g, Tween 40 0.1 g, Tris 50 mmol, diethylenetriaminepentaacetic acid 20 μmol, and trichloroacetic acid 0.25 mol. The final trichloroacetic acid concentration in the well during the assay, 0.2 mol/L, sufficed to displace cortisol from endogenous binding proteins.

Multiple-ion-detection mass spectrometry. For the mass fragmentography we used a modification of the procedure of Björkhem et al. (12). We prepared serum samples by adding 100 ng of prednisolone in 50 μL of ethanol to 0.5 mL of serum. This mixture was extracted with 5 mL of methylene chloride and the organic phase was evaporated under a stream of nitrogen at 70°C. To the residue we added 50 μL of a 100 g/L solution of methoxylamine hydrochloride in pyridine and allowed the mixture to stand at 70°C for 15 min before evaporating the solvent under a stream of nitrogen. The residue was treated with 50 μL of trimethylsilylimidazole at 100°C for 2 h or with 50 μL of pentafluoropropionic anhydride at 70°C for 20 min. Excess reagent was removed by evaporation and the residue was extracted with hexane. The hexane phase was separated, the hexane was evaporated, and the residue was redissolved in 25 μL of hexane.

The serum samples were analyzed by gas chromatography-mass spectrometry, for which we used an LKB 9000 instrument equipped with a 7-m capillary column coated with SE-30. The carrier gas was helium and the column temperature 250°C. The electron energy was set to 20 eV and the trap current to 60 μA. In the case of trimethylsilylimidazole the first channel of the multiple-ion-detection unit focused on the ion at m/e 603 and the second at m/e 605; the settings for pentafluoropropionic derivatives were 615 and 617, respectively.

Time-resolved fluoroimmunoassay. We pipetted 20 μL of standards or serum specimens into the strip wells, then added 100 μL of Eu-labeled anti-cortisol IgG in assay buffer containing trichloroacetic acid and incubated the mixture at room temperature for 2 h. The immunoreaction was stopped by washing the wells (Nunc Immunowash 12; A/S Nunc, Roskilde, Denmark) three times with a solution containing, per liter, 9 g of NaCl, 500 mg of NaN3, and 250 mg of Tween 20.

After the immunoreaction was complete, the Eu bound to the solid phase was dissociated by the addition of an enhancement solution. We measured the fluorescence of the 2-naphthyltrifluoroacetone chelate (3) with a time-resolved fluorometer capable of single photon counting (1230 Arcus; LKB-Wallac, Turku, Finland) (2).

Results

Optimization of the Assay

Effect of the amount of immobilized cortisol and label on the standard curve. The assay for cortisol was optimized both with respect to the amount of immobilized cortisol-3-CMO-ovalbumin on the solid phase and the amount of Eu-labeled antibody, taking into consideration the required working range. Figure 1 shows the effect of the concentration of the coating solution of cortisol-3-CMO-ovalbumin on the replacement for different concentrations of standard when the concentration of labeled anti-cortisol IgG was kept constant. The lower the concentration used to prepare the solid phase, the steeper the curve and the better the replacement. Figure 2 shows the corresponding set of curves obtained when the amount of the labeled antibody was varied and the amount of cortisol-4-CMO-ovalbumin on the solid phase was kept constant. The less labeled antibody used, the better the replacement. In further experiments we used a coating concentration of 0.5 μg/mL and 200 ng of labeled anti-cortisol IgG per well. The final choice was based on the signal intensity and replacement efficiency obtained.

Displacement of cortisol from endogenous binding proteins. For this we used a novel procedure based on the chaotropic agent trichloroacetic acid. Figure 3 shows the effect of various concentrations of trichloroacetic acid on cortisol binding in the presence of different concentrations of cortisol. Displacement became constant at a trichloroacetic acid concentration of 0.2 mol/L. Thus we used this concentration in the assay to displace cortisol from binding proteins. The decrease in signal caused by the use of trichloroacetic acid in the assay can be counteracted by adding somewhat more labeled antibody to the system (see Figure 2).

Assay kinetics. We investigated the kinetics of the antigen–antibody reaction at room temperature in the presence of different amounts of cortisol. A 2-h incubation time was
chosen, even though the reaction does not reach equilibrium by then, because the time can easily be controlled and reproduced. No effect of variations in sample-handling time was seen in analyses of 300 wells in one run. Patients' serum samples followed the same reaction kinetics; therefore, we chose an incubation time of 2 h for the assay.

Assay Performance

A typical standard curve of time-resolved fluoroimmunoassay of cortisol is shown in Figure 4. The sensitivity (2 SD) of the assay, as calculated from 12 replicates of the zero standard, was about 3 nmol/L. A replacement of almost 90% is obtained with the highest standard. The precision profile of the assay was determined from 12 replicates, the CVs at each concentration of standard—10, 110, 200, 500, 1000, and 2000 nmol/L—being 27.9, 6.2, 6.7, 7.3, 6.4, and 5.3%, respectively.

To test the linearity of the time-resolved fluoroimmunoassay of cortisol, we added three different volumes of patients' sera (20, 10, and 5 μL) without correcting for the change in assay volume. Very good linearity was obtained for all five specimens examined, the cortisol concentrations of which ranged from 305 to 1245 nmol/L.

Analytical recovery of the assay was assessed by adding three different amounts of cortisol (to give final exogenous concentrations of 90, 220, and 430 nmol/L) to five different patients' sera with endogenous cortisol concentrations of 102 to 853 nmol/L. The percent recovery was calculated after subtracting the endogenous cortisol concentration from the experimentally determined amount. Recoveries ranged from 80 to 136% (n = 15, x̄ = 98.9%, SD = 15.9%).

The specificity of the Eu-labeled cortisol antisera was tested against several steroids. Cross reactions (50% inhibition) were 100% for cortisol, 21.9% for prednisolone, 14.2% for 11-deoxycortisol, 5.2% for prednisone, 4.6% for 17α-hydroxyprogesterone, 4.3% for cortisone, 2.4% for dexamethasone, 1.2% for corticosterone and 11-dehydrocorticosterone, 1.1% for deoxycorticosterone, 0.7% for progesterone, 0.04% for spironolactone, 0.03% for dehydroisoandrosterone, and <0.01% for estrone and estriol.

Inter-method comparison and normal reference interval. We assayed 72 normal and abnormal serum samples by both our assay (TR-FIA) and radioimmunoassay (RIA). The linear regression equation was (TR-FIA) = 3.6 + 1.09 (RIA) and the correlation coefficient (r) was 0.93. Comparison of our assay and multiple ion-detection mass spectrometry (MID-MS) for the analysis of 27 samples gave the linear regression equation: (TR-FIA) = −9.1 + 1.05 (MID-MS), r = 0.95.

Serum cortisol was measured in specimens collected in the morning from 94 apparently healthy staff members (41 women, 53 men), ages 18 to 54 years. The mean concentration of cortisol in serum was 478 nmol/L (SD 165). A log-normal cumulative probability plot of the data gave a reference interval of 250–850 nmol/L.

Discussion

The performance characteristics of the new cortisol assay meet all the criteria for a reliable and accurate test of cortisol in serum. The potential sensitivity of the time-resolved fluorescence technique with europium labeling is not fully exploited in the serum cortisol assay, having been instead optimized for the clinically important concentration range. However, the specific activity of the label is extremely high (2), and all prerequisites exist for the development of highly sensitive hapten assays in which the activity of the label causes no restrictions.

The displacement of cortisol from endogenous binding proteins should ideally be carried out under conditions that do not interfere with the immunoassay. In direct nonextraction methods this is usually achieved by treatment with heat (13), protease (14), low pH (15, 16), 8-anilino-1-naphthalene sulfonic acid (17, 18), or salicylate (19, 20). The europium label dissociates from the labeled antibody at low pH, which consequently excludes this from the displacement method of choice. Lack of experimental success with 8-anilino-1-naphthalene sulfonic acid and salicylate at neutral pH led us to develop a completely new approach based on the use of trichloroacetic acid at neutral pH. The inactivation of the binding proteins and a concomitant displacement of cortisol are achieved under conditions that do not affect the performance of the assay. This novel displacement agent also functions well in other direct hapten assays in which binding proteins interfere (to be published).

The results obtained with the antibody-labeled assay of cortisol demonstrate that time-resolved fluorometry in combination with the europium label offers a potential nonisotopic alternative to hapten assays. Moreover, because of the
high specific activity of the label, the technique can be used in assays that require a sensitivity difficult to attain with other nonisotopic alternatives.

We are grateful to the Wallac Organic Chemistry Laboratory for synthesis of the cortisol derivatives, to Mrs. Teija Ristelä for secretarial assistance, and Mrs. Arja Koikkinen for the illustrations.

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CLIN. CHEM. 31/10, 1734–1736 (1985)

Oligoclonal Banding in Cerebrospinal Fluid Assessed by Electrophoresis on Agarose after Centrifugal Sample Concentration through a Microconcentrator Membrane

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We describe use of a microconcentrator membrane with a 30,000-Da cutoff for treatment of cerebrospinal fluid (CSF) specimens before detection of oligoclonal bands by electrophoresis on agarose. After centrifugation at 2000 × g for 25 min, 0.5-, 1.0-, and 2.0-mL aliquots of CSF were concentrated 15-, 25-, and 40-fold. Analytical recovery of immunoglobulins G and A from the microconcentrators was about 90% (CV 5–8%). We found good correlation between results by this method and by a silver-stain procedure in a study comparing oligoclonal banding in CSF from multiple sclerosis and control patients. After 40-fold concentration of 2 mL of CSF, 2 mg of immunoglobulin G per liter can be detected, because the analytical sensitivity of the electrophoresis is 80 mg/L.

Additional Keyphrases: multiple sclerosis • silver-stain method compared

Electrophoretic detection of oligoclonal bands was originally described in cerebrospinal fluid (CSF) from multiple sclerosis (MS) patients (1–3). Although much of the work concerning oligoclonal banding has focused on the laboratory-assisted diagnosis of MS, these bands have also been reported in association with systemic lupus erythematosus

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Received June 17, 1985; accepted July 19, 1985.