Europium and Samarium as Labels in Time-Resolved Immunofluorometric Assay of Follitropin

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This time-resolved immunofluorometric assay for human follitropin involves use of europium- or samarium-labeled monoclonal antibodies, with an average incorporation ratio of 3 mol of Eu3+ or Sm3+ per mole of antibody. These lanthanide ions are bound to the antibody molecules by means of the anhydride of diethylenetriaminepentaacetic acid. The solid-phase antibody is immobilized inside polystyrene tubes in which plasma samples were assayed in a one-step procedure. After incubation, the fluorescence intensity of Eu3+ or Sm3+ label is measured by time-resolved fluorometry, with a nitrogen laser as the pulsed excitation source. The sensitivity of the assay is largely better with Eu3+ than with Sm3+ because of the difference in their intrinsic luminescence properties. Results obtained with the proposed methods correlated well with those by an immunoradiometric method.

Additional Keyphrases: lanthanide labels · monoclonal antibodies · immunoradiometric assay compared

Recently, there has been considerable interest in development of new nonisotopic immunoassays. The methods that are most efficient in terms of both specificity and sensitivity make use of luminescent markers, particularly certain rare-earth chelates. Because of their long fluorescence lifetime (10−9 to 10−12 s), these labels can be detected in very low concentrations by means of a pulsed excitation source coupled to a time-resolved luminescence detection system. Such a fluorometric technique enables the total rejection of the optical background, which is the main limiting factor of the sensitivity in conventional fluorometry.

Several time-resolved immunofluorometric assays (TRIFMA)4 involving Eu3+-labeled antibodies have been described for determinations of human choriongonadotropin (1), pancreatic phospholipase A2 (2), hepatitis B surface antigen (3), cortisol (4), thyrotropin (5), and insulin (6). In these techniques, solid-phase two-site assays and solid-phase competitive assays, the bound fraction of Eu3+ label is extracted and its fluorescence is measured by time-resolved fluorometry with a xenon flashtube excitation (7). More recently, Déchaud et al. (8) have reported a laser-excited TRIFMA for human prolactin with Eu3+ as the lanthanide label.

Among the other trivalent lanthanide ions potentially usable as markers, only Tb3+ has received attention (9), having been applied to IgG determination by competitive immunoassay (10). Unfortunately, terbium has a major drawback for fluorimunoassay: its excitation wavelength requires the use of quartz cuvettes instead of polystyrene tubes (9). Sm3+, however, despite its well-known luminescence properties in solution (11, 12), has never been used as suggested as a label for TRIFMA.

We describe here a laser-excited time-resolved immuno fluorometric assay for human follitropin (FSH) in plasma involving two different monoclonal antibodies against two separate determinants. Europium or samarium is attached to one of these monoclonal antibodies by using the anhydride of diethylenetriaminepentaacetic acid (DTPA). For either label the same extraction solution is used after the assays (see Results) to allow the respective fluorescent chelates to form. Results obtained by Eu-TRIFMA and Sm TRIFMA are compared with each other and with those by conventional immunoradiometric assay (IRMA).

Materials and Methods

Instrumentation

The time-resolved fluorometer developed in our laboratory, described previously (8), is based on a nitrogen laser excitation at 337.1 nm with a repetition rate of 50 Hz and a pulse duration of 8 ns. The main emission bands of Eu3+ and Sm3+ are selected with interference filters (613 nm, 10-nm bandwidth, 65% peak transmission, for europium; 643 nm, 11-nm bandwidth, 67% peak transmission, for samarium). The signal is processed in the photon-counting mode. With either label, samples are analyzed in polystyrene tubes (useful volume: 300 μL).

Reagents

Chemicals. DTPA anhydride and human gamma globulin purified from Cohn Fractions II and III were from Sigma Chemical Co., St. Louis, MO 63178. Tri-n-octylphosphine oxide was purchased from Merck AG, Darmstadt, F.R.G. Europium chloride (EuCl3) and samarium chloride (SmCl3) were obtained from Aldrich-Chemie SARL, Strasbourg, France. β-Naphthyltrifluoroacetone was synthesized according to the method described by Reid and Calvin (13).

Monoclonal antibodies. FSH standards calibrated against the Medical Research Council 2nd International Reference Preparation 78/549 of human FSH, polystyrene tubes coated with the first monoclonal antibody to human FSH, and the second monoclonal antibody for lanthanide labeling were kindly donated by BioMérieux, Charbonnières-les-Bains, France. IRMA kits for measuring FSH (125I-T4FSH COAT-RIA, cat. no. 6 6730) were purchased from BioMérieux.

Reagent solutions. The assay buffer contained, per liter, 5 mmol of Tris HCl (pH 7.75), 9 g of NaCl, 5 g of bovine serum albumin, 0.5 g of NaN3, 0.5 g of gamma-globulin, 25 μmol of...
EDTA, and 0.1 g of Tween 40 polyoxyethylene (40) sorbitan monopalmitate surfactant. The wash solution was doubly distilled water containing 0.22 g of Tween 20 polyoxyethylene (20) sorbitan monolaurate surfactant per liter, with the pH adjusted to 8.0 by adding Tris. The extraction solution for fluorescence measurements of Eu\(^{3+}\) and Sm\(^{3+}\) was modified from that of Hemmilä et al. (9); it contained 10 μmol of β-naphthoyltrifluoroacetone, 40 μmol of tri-n-octylphosphine oxide, and 0.1 mL of Triton X-100 surfactant per liter of doubly distilled water, and was adjusted to pH 3.5 with citric acid.

**Procedures**

**Preparation of labeled antibodies.** To bind Eu\(^{3+}\) or Sm\(^{3+}\) ions to antibodies, we used the method previously described for prolactin TRIFMA (8), a modification of the method reported by Krejcíre and Tucker (14). Add 100 μL of the solution of the second monoclonal antibody (about 10 mg/mL) dropwise to 260 μg of DTPA anhydride and adjust the pH to 7.0 with dilute NaOH. After vortex-mixing for 1 min, incubate the solution for 30 min at room temperature. Remove the excess DTPA by two consecutive dialyses against citrate buffer (50 mmol/L, pH 6.0), then add 25 μL of a 33 mmol/L solution of europium or samarium chloride in citrate buffer to the purified DTPA-coupled antibody. Incubate for 1 h at room temperature, with gentle agitation. Purify the lanthanide-labeled antibody by size-exclusion chromatography (we used a 22 × 1.5 cm column of Sephadex G 50, from Pharmacia, Uppsala, Sweden). In our hands, the incorporation ratio of europium or samarium to antibody was about 3 mol/mol.

**Sample collection.** The studied population consisted of 34 women and 17 men, ages 15 to 55 years. Of these, 32 were healthy and 19 presented with gonadal disorders. Blood was sampled under basal or stimulated conditions (under a gonadotropin-stimulation test with lutein). After centrifugation, plasmas were stored in aliquots at −20 °C until assayed for FSH.

**Time-resolved immunofluorometric assays (Eu-TRIFMA and Sm-TRIFMA).** Transfer 100 μL of reconstituted standards or plasma samples into the coated tubes in duplicate. Add 200 μL of assay buffer containing 0.75 pmol of second antibody, then incubate for 3 h at room temperature with continuous gentle agitation. Wash the contents of each tube carefully four times with 500 μL of wash solution, then add 500 μL of extraction solution to allow the fluorescent chelates to form. After agitation for 30 min, measure the fluorescence by photon counting with the time-resolved fluorometer under the following conditions: for the Eu\(^{3+}\) label, wavelength = 613 nm, delay time = 200 μs, gate time = 1 ms, integration over 500 cycles; for the Sm\(^{3+}\) label, wavelength = 643 nm, delay time = 50 μs, gate time = 100 μs, integration over 1000 cycles.

The IRMA technique was performed under the same conditions as the TRIFMA, with some modifications: after the 3-h agitation, we washed the tubes twice and directly measured the bound radioactivity with a gamma counter.

**Results**

**Europium and samarium luminescence analysis.** After the assays, the Eu\(^{3+}\) or Sm\(^{3+}\) ions used as photoluminescent markers must be displaced from the antibody molecules into a fluorescent chelate, to be detectable with enough sensitivity (9). Figure 1 shows the emission spectra (excitation wavelength 337 nm) of europium and samarium chelated by β-naphthoyltrifluoroacetone in the extraction solution used for the TRIFMA. The strongest emission peaks are at 613 nm for the Eu complex, 643 nm for the Sm complex. These emission bands arise from the electron transitions described in Figure 1. Determination of the emission lifetimes (Figure 2) led us to select the following optimum conditions for the time-resolved detection: 200 μs delay time and 1 ms gate time for europium; 50 μs delay time and 100 μs gate time for samarium. The detection limits, calculated from the average residual background + 3 SD, were 3 × 10⁻¹⁸ mol for europium and 2 × 10⁻¹⁸ mol for samarium in 300 μL of the extraction solution.

**Standard curves.** Figure 3 shows two typical calibration curves for human FSH obtained with the two labels. In both cases, the response in fluorescence intensity, corrected for that from the zero sample signal, is strictly linear over the whole range of standardization. With the europium label the detection limit for human FSH, defined as the concentration giving the zero signal + 3 SD (calculated from 12 replicates), was 0.13 int. unit/L in plasma. The weaker emission signal of the samarium label means its detection limit for FSH is about 10-fold higher.

**Figures**

**Fig. 1.** Normalized fluorescence spectra of Eu\(^{3+}\) and Sm\(^{3+}\) chelated by β-naphthoyltrifluoroacetone in the extraction solution

Excitation wavelength, 337.1 nm; delay time, 10 μs; gate time, 20 μs.

A.U., arbitrary units

**Fig. 2.** Normalized luminescence decay curves for Eu\(^{3+}\) and Sm\(^{3+}\) obtained at the wavelengths of their emission maximum

Chelating agent, β-naphthoyltrifluoroacetone; gate time, 2 μs.
The dashed vertical lines indicate the respective limits of detection for FSH.

**Discussion**

Although europium is the choice marker for immunoassay based on time-resolved luminescence, samarium can be also used in assays that do not require great sensitivity. Moreover, the lanthanide labeling method applied here, and previously described for prolactin immunoassay (6), is simple and rapid owing to the use of a commercially available reagent (DTPA anhydrate). The advantages of excitation of rare-earth chelates by a laser source instead of a discharge lamp have been already discussed (6) in terms of efficiency, stability, and power adaptability.

For the Eu-TRIFMA of human FSH, our results are similar to those obtained by the IRMA we routinely used. The limit of detection (about 0.1 int. unit/L) is much better than with usual competitive radioimmunoassays. The response is linear from 0 to 135 int. units/L, with low CVs in the usual concentration range. The main advantages of Eu-TRIFMA over IRMA are the use of a nonradioisotopic label and the shorter counting time per tube (1 s can be sufficient). The principal drawback is that the assay tubes must be carefully washed several times before the luminescent signal is measured.

Using Sm$^{3+}$ as the marker of the second monoclonal antibody diminishes the advantages of the TRIFMA somewhat, because the quantum yield for samarium photoluminescence is lower than that for europium. The counting rates are therefore substantially weaker and, despite use of a longer counting time, the sensitivity and the precision provided by the Sm-TRIFMA for human FSH are not yet adequate. However, results can be significantly improved by increasing the Sm$^{3+}$ labeling yield. By using a coupling reaction with isothiocyanatobenzyl-diethylenetriamine tetracetate (6), or with isothiocyanatophenyl-EDTA (9), corresponding to a modification of the method reported by Sundberg et al. (15), incorporation ratios >10 mol/mol can be obtained, without affecting immunoreactivity (6), contrary to strong DTPA coupling (8). Moreover, given the spectral characteristics of Sm$^{3+}$ and Eu$^{3+}$, our findings may open the possibility of double labeling, to assay two different hormones simultaneously in the same tube—e.g., to study follitropin and lutropin in abnormalities of the reproductive system of men and women. This objective is realistic for several reasons: (a) Sm$^{3+}$ and Eu$^{3+}$ are excitable at the same wavelength (about 340 nm) in polystyrene tubes; (b) they are extracted and chelated in the same medium; (c) their emission maxima are separated by 30 nm in the long-wavelength region of the spectrum; (d) their emission lifetimes differ significantly. The first two points are not true for Tb$^{3+}$; consequently, the prospect of double labeling is more credible with Eu$^{3+}$/Sm$^{3+}$ than with the Eu$^{3+}$/Tb$^{3+}$ pair previously suggested (9).

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References