Double-Label Time-Resolved Immunofluorometry of Lutropin and Follitropin in Serum
Ilkka Hemmilä, Sirkku Holttin, Kim Pettersson, and Timo Lövgren

We describe a procedure for the simultaneous immunofluorometric assay of lutropin and follitropin in human serum, based on the use of monoclonal antibodies and of the fluorescent lanthanides Eu³⁺ and Tb³⁺. The α-chain-specific antibody was used as a common capture antibody on the surfaces of microtiter strips. The anti-β-follitropin antibody was labeled with Tb³⁺, the anti-β-lutropin antibody with Eu³⁺. After the immunoreactions had taken place, the bound fractions of the labels were dissociated in a fluorescence enhancement solution of pivaloyl trifluoroacetone, triocetylphosphine oxide, and Triton X-100 surfactant. In this solution both lanthanides can be measured successively with a time-resolved fluorometer. The detection limit of the assay is 0.1 int. unit/L for lutropin and 1 int. unit/L for follitropin. Results correlated well with those by commercial immunofluorometric assays and radioimmunoassays.

**Additional Keyphrases:** europium · terbium · peptide hormones · monoclonal antibodies

Assessment of follitropin and lutropin is often required when pathological abnormalities of the male and female reproductive systems are being investigated. Besides several commercial immunoassay kits for monitoring these analytes individually, some manufacturers have also recently produced double-label radioimmunoassay kits for simultaneously measuring these hormones, using ⁵⁷Co-labeled lutropin and ¹²⁵I-labeled follitropin (1).

Immunofluorometric assays have also been developed for lutropin (2) and follitropin (Pettersson and Söderholm, manuscript in preparation) with use of Eu³⁺-labeled monoclonal antibodies. The dissociative fluorescence enhancement solution used in these assays contains the ligand, 2-naphthyl trifluoroacetone, which absorbs excitation light (3). This ligand is also applicable to the excitation of Sm³⁺, which has been suggested as counterpart to Eu³⁺ in a double-label system (4).

Actually, Eu³⁺ and Tb³⁺ form the most efficient fluorescent chelates (5), but the enhancement solution optimized for Eu³⁺ detection cannot be used for Tb³⁺ (6). Instead, aliphatic β-diketones can have enough energy in the excited triplet state for the excitation of both Eu³⁺ and Tb³⁺ in the same solution; moreover, the resulting emissions can be easily distinguished from each other (6). In this study we labeled specific monoclonal antibodies against the β-subunits of the hormones with Eu³⁺ and Tb³⁺, and tested various assay combinations. After determining the optimal combination, we analyzed serum samples for lutropin and follitropin, using one set of standards containing both analytes and using a common fluorescence enhancement solution for measuring Eu³⁺ and Tb³⁺ successively in a time-resolved fluorometer.

Materials and Methods

**Reagents.** EuCl₃ and TbCl₃ were obtained from Aldrich Chemical Co., Milwaukee, WI 53201. The β-diketone, pivaloyl trifluoroacetone (1,1,1-trifluoro-5,5-dimethyl-2,4-hexadiene) was synthesized by Claisen condensation from pivalylmethylene and ethyl ester of trifluoroacetic acid, with NaH as the condensing agent (7).

The monoclonal antibodies to α- and β-follitropin were obtained from LKB-Wallac, Turku, Finland, and anti-lutropin clones 526 and 543 from Clonatec, Paris, France. The lutropin and follitropin standards, calibrated against the WHO standard preparations (lutropin, 1st IRP 68/40; follitropin, 2nd IRP 78/549), were obtained from Boehringer, Mannheim, F.R.G., and diluted to give final concentrations of 1, 4, 16, 64, and 256 int. units/L with the Delfia™ assay buffer (LKB-Wallac). This buffer is Tris HCl buffer, 50 mmol/L, pH 7.75, containing, per liter, 9 g of NaCl, 0.5 g of NaN₃, 5 g of bovine serum albumin, 0.5 g of bovine globulin, and 0.1 mL of Tween 40. The Delfia immunofluorometric assay kits for lutropin and follitropin were also obtained from LKB-Wallac.

The serum specimens, already analyzed with a commercial RIA (Farmos Diagnostica, Turku, Finland), were obtained from the University Central Hospital of Turku.

**Instruments.** For the spectral analysis of the fluorescent chelates we used a Model LS5 luminescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT), in the phosphorescence mode. Eu³⁺ and Tb³⁺ were quantified with a Model 1230 Arcus time-resolved fluorometer (LKB-Wallac) equipped with filters for Eu³⁺ (613 nm) and Tb³⁺ (545 nm).

**Labeling the antibodies.** To label the monoclonal antibodies with Eu³⁺ and Tb³⁺, we used N⁵-(p-isothiocyanatobenzyl)-diethylenetriamine-N¹,N²,N⁵,N⁶-tetraacetic acid as described earlier (8). Conjugations were performed either at pH 9.8 or 8.5 with a 100- to 1000-fold molar excess of the reagents. The incorporation yields varied between four and 25 metal (III) ions per molecule of IgG.

**Simultaneous immunofluorometric assay.** We dispensed 100 μL of samples or standards and 100 μL of assay buffer onto microtitration strips (Eflab, Helsinki, Finland) coated with the monoclonal antibodies to α-subunit. After a 2-h incubation at room temperature with continuous gentle shaking we washed the wells three times with wash solution, containing, per liter, 9 g of NaCl, 0.2 mL of Tween 20, and 1 g of a preservative, "Germall-II" (Sutton Laboratories Inc., Chatham, NJ). We then added the labeled antibodies, 500 ng of both Eu³⁺-labeled anti-β-lutropin and Tb³⁺-labeled anti-β-follitropin in 200 μL of assay buffer. After a further 1-h incubation with shaking we washed the wells six times with the wash solution. We dissociated the bound fractions of metals by adding to each well 200 μL of the enhancement solution (200 μmol of pivaloyl trifluoroacetone, 100 μmol of triocetylphosphine oxide, and 5 mL of Triton X-100 per liter of acetate buffer, 0.1 mol/L, pH 4.0). After shaking the strips for 10 min we measured the fluorences with an Arcus fluorometer. The measurement setup for Eu³⁺ was as follows: cycling time 2 ms, delay time...
Results

Fluorescence Detection of Eu\(^{3+}\) and Tb\(^{3+}\)

For simultaneous measuring of the metals in the same solution we applied a \(\beta\)-diketone-based fluorescence enhancement solution (6). Use of slightly higher reagent concentrations than those used before kept the signals stable, even at different Eu\(^{3+}\)/Tb\(^{3+}\) ratios. Because low pH is needed for metal ion dissociation (3), we determined the lowest pH that still gave a near-optimal fluorescence value for both metals. For Eu\(^{3+}\) the fluorescence was practically stable at pHs >4.0, decreasing steeply at pHs <4.0, whereas Tb\(^{3+}\) fluorescence showed a maximum at a pH of about 4.0 to 4.5.

Figure 1 shows the emission spectra of Eu\(^{3+}\) and Tb\(^{3+}\) chelated by pivaloyltrifluoroacetone in the enhancement solution and excited at 305 nm, which is the absorption maximum of the \(\beta\)-diketone. The strongest emission peaks were at 614 nm for Eu\(^{3+}\) and 544 nm for Tb\(^{3+}\). The fluorescence decay times of Eu\(^{3+}\) and Tb\(^{3+}\) in that solution were 1020 \(\mu\)s and 148 \(\mu\)s, respectively (Table 1). Because of the large difference in the decay times, we optimized various measurement setups for the metals. This also minimizes spectral overlapping caused by the minor emission peak of Tb\(^{3+}\) at 621 nm near the main emission peak of Eu\(^{3+}\) at 614 nm (Figure 1). There was 0.01% fluorescence interference by Eu\(^{3+}\) with Tb\(^{3+}\) and 1% by Tb\(^{3+}\) with Eu\(^{3+}\), which proved to be negligible. The detection limit for Eu\(^{3+}\) was near 1 pmol/L and for Tb\(^{3+}\) 40 pmol/L when defined as the concentration of the lanthanides giving a specific signal equal to the background (Table 1).

Double-Label Assay of Lutropin and Follitropin

According to the assay combination tests, performed with Eu\(^{3+}\)-labeled antibodies, the follitropin assay was slightly more sensitive that the lutropin assay. On the other hand, the measurement of Tb\(^{3+}\)-labeled antibodies was less sensitive than the measurement of Eu\(^{3+}\)-labeled antibodies, because of the lower signal and higher background obtained in Tb\(^{3+}\) detection (Table 1). Thus we constructed a double-label assay by using Tb\(^{3+}\)-labeled anti-\(\beta\)-follitropin, Eu\(^{3+}\)-labeled anti-\(\alpha\)-subunit antibodies on the solid phase.

Figure 2 shows typical dose–response curves and Figure 3 the precision profiles calculated from data on 12 replicates of the standards. After subtracting the zero-standard signal, we found the standard curves to be linear from 1 to 256 int. units/L. The capacity of the capture antibody was sufficient for the simultaneous binding of the most concentrated standards from both hormones. The detection limits of the assays were 0.1 int. unit/L for lutropin and 1.0 int. unit/L for

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Table 1. Fluorescence Properties of Eu\(^{3+}\) and Tb\(^{3+}\) Chelates

<table>
<thead>
<tr>
<th>Metal</th>
<th>Excitation (max), (\text{nm})</th>
<th>Emission (max), (\text{nm})</th>
<th>Decay time, (\mu\text{s})</th>
<th>Background signal, counts/s</th>
<th>Detection limit, pmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eu</td>
<td>308</td>
<td>614</td>
<td>1020</td>
<td>420</td>
<td>1</td>
</tr>
<tr>
<td>Tb</td>
<td>307</td>
<td>544</td>
<td>148</td>
<td>4490</td>
<td>40</td>
</tr>
</tbody>
</table>

*The fluorescence properties of Eu\(^{3+}\) and Tb\(^{3+}\) chelates with pivaloyltrifluoroacetone were measured with a Perkin-Elmer LS5 spectrofluorometer (excitation and emission wavelengths and decay times) and with an Acuron fluorometer (background and sensitivity).
follitropin, calculated as that concentration of hormones giving a signal equal to the signal of the zero sample plus three standard deviations.

We tested the specificity of the assay by measuring the cross reactivities of the labeled antibodies against lutropin, follitropin, and chorionic gonadotropin, using anti-α-subunit antibodies on the solid phase. The results are presented in Table 2.

We compared results by the present assays with those from analogous Delfias and from RIAs by measuring lutropin and follitropin in clinical samples by all the methods. The results inter-correlated well (Table 3). However, the double-label assay of lutropin gave 50% higher values than the Delfia—which is obviously ascribable to the β-subunit-specific monoclones chosen. The current antibody combination gave the same lutropin values for samples as those obtained with radioimmunoassay.

Discussion

The fluorescent lanthanides (Eu³⁺, Tb³⁺, Sm³⁺, and Dy³⁺) are ideal candidates for double-label or even multiple-parameter assays, owing to their characteristic narrow-banded emission lines. Their emissions are clearly distinguishable from each other with respect to both to wavelengths and lifetimes, and this can be exploited in time-resolved fluorometry. The spill-over between the measurements of Eu³⁺ and Tb³⁺ is less than 1% as compared to 3% between ⁵⁷Co and ¹²⁵I currently used in double-label RIAs (1).

Eu³⁺ is the lanthanide most commonly used in time-resolved fluoroimmunoassays (9) and it is also applied in the Delfia assay of lutropin and follitropin. The fluorescent chelates of Eu³⁺ have high quantum yields, and the measurement is not sensitive to background interference (3). The problem in double-label systems arises from the lower fluorescence and higher backgrounds in the detection of the other lanthanides besides Eu³⁺. In a recent paper (4), Sm³⁺ was used as an alternative to Eu³⁺ in an assay of follitropin, and the Sm³⁺ and Eu³⁺ pair was suggested for double-label assays. Sm³⁺ forms fluorescent chelates with aromatic β-diketones and can be measured in a fluorescence enhancement solution already optimized for Eu³⁺ (3, 10). It has a long-wavelength emission at 643 nm, well separated from disturbing backgrounds. On the other hand, it has lower quantum yields (10, 11) and shorter decay times, around 50 μs, than do the Eu³⁺ or Tb³⁺ chelates (12).

Tb³⁺ and Eu³⁺ form another pair suggested for double-label systems (5). Tb³⁺ chelates often have reasonably high fluorescence quantum yields and long decay times (11, 12). The emission at 543–545 nm is, however, more sensitive to interferences derived from plastic materials (6), and its fluorometry requires shorter excitation wavelengths and thus cannot be performed by using the 2-naphthyltrifluoroacetone of the Eu³⁺ enhancement solution. Replacement of the naphthyl derivative with a fluorinated aliphatic β-diketone such as pivaloyltrifluoroacetone produces a common fluorescence enhancement solution suitable for use with Eu³⁺, Tb³⁺, and Sm³⁺ (6, 11).

Because of the lower sensitivity in the Tb³⁺ detection, the double-label assay utilizes higher sample volumes (100 μL) and concentrations of labeled antibodies per assay well (2500 ng/mL), higher than those used in the single-label assay (Delfia): 25 μL, 250 ng/mL. The resulting assay sensitivities were 0.1 int. unit/L for lutropin (Eu³⁺) and 1.0 int. unit/L for follitropin (Tb³⁺), which were somewhat inferior to those obtained with the single-label assay (0.12 and 0.05 int. unit/L) but still considerably higher than those obtained with commercial double-label radioimmunoassays: 2–4.4 int. units/L for lutropin and 1.5–3.6 int. units/L for follitropin (1).

The present work demonstrates the utility of the pair of the fluorescent lanthanide ions Eu³⁺ and Tb³⁺ in simultaneous double-label immunoassays. In addition to the lutropin–follitropin pair, this technique can be applied to other analyses where two analytes are frequently measured in the same sample, e.g. thyroxin and thyrotropin.

References