Homogeneous Time-Resolved Fluoroimmunoassay of Thyroxin in Serum

Ilkka Hemmilä, Outi Meilinmäki, Heikki Mikola, and Timo Lövgren

We describe a rapid, simple noneparation fluoroimmunoassay for determination of thyroxin in serum. The assay is based on the labeling of thyroxin directly with a fluorescent europium chelate, the fluorescence of which is quenched on binding to an antithyroxin antibody. With the assay buffer we used, maximum quenching is 90%. The rapid achievement of equilibrium in the assay solution, regardless of the sequence of reagent additions, allows fast measurement of thyroxin. Precision was good (CV <5%) within the clinical range for total thyroxin (50–300 mmol/L), and results correlated well with those by a commercial radioimmunoassay.

Additional Keyphrases: europium chelates · monoclonal antibodies

The development of europium labeling reagents and a fluorescence-detection method based on europium dissociation (1) has opened new possibilities for fluoroimmunoassay (FIA) when combined with the principle of time-resolved fluorometry (2). Especially where high sensitivity and a wide measurement range are required, a commercial time-resolved fluorescence immunoassay, DELFIA*, has gained wide application (3, 4). This method has been applied to competitive assays, such as those for cortisol (5), digoxin (6), and thyroxin (7), which require a separation step and washing, and the DELFIA assays also require an additional fluorescence-enhancement step.

In addition to the Eu** labeling reagents used for DELFIA,

Wallac Chemical Laboratories, P.O. Box 10, SF-20101 Turku, Finland.
Received February 17, 1988; accepted June 27, 1988.

which produce practically nonfluorescent conjugates, a few stable and fluorescent chelates of lanthanides were introduced and used as labels in time-resolved FIA (8–10). Some of the chelate structures show such sensitivity to environmental changes that they can be exploited in homogeneous assays (10). One such fluorescent europium chelate, developed at Wallac and coupled to thyroxin, was used in this study to evaluate the usefulness of these chelates for homogeneous time-resolved FIA.

Materials and Methods
Reagents. The monoclonal antibody against thyroxin, produced in ascites, was obtained from Cambridge Medical Diagnostics, Billerica, MA 01865. The same antibodies are supplied in DELFIA kits. The determined cross reactivities against triiodothyronine analogs varied between 14 and 3% cross reactivities against d-thyroxin were 56% and against other analogs they were <0.15%.

The thyroxin standards from Wallac Oy were prepared in charcoal-treated normal human serum (Wolf Brandenberger, Zurich, Switzerland). The clinical serum samples, analyzed with a commercial thyroxin RIA (Farmos Diagnostica, Turku, Finland) were obtained from the University Central Hospital of Turku. The fluorescence-enhancement solution used for total Eu** measurements was obtained from Wallac Oy.

The pH 5.5 assay buffer contained, per liter, 50 mmol of phthalate buffer, 9 g of NaCl, 2 g of thimerosal (Sigma Chemical Co., St. Louis, MO 63178), and 10 g of bovine serum albumin.

Labeling of thyroxin. Disuccinimidyl suberate, prepared from suberic acid and N-hydroxysuccinimide in dioxane, was coupled to l-thyroxin in anhydrous dimethylformamide
solution. A fluorescent europium chelate (W-1174, not yet commercially available) from the Wallac Organic Chemistry Laboratory, was coupled to L-thyroxin by adding an aqueous solution of the chelate to a dioxane solution containing the succinimidylalberic acid amide derivative of L-thyroxin. The concentration of the tracer was calculated according to the concentration of Eu\(^{3+}\), which was measured fluorometrically in fluorescence-enhancement solution (1).

**Fluorimmunoassay.** In the assay, 20-\(\mu\)L aliquots of serum samples or standards in duplicates are dispensed into microtitration strip wells (Etabl, Helsinki, Finland), and 200 \(\mu\)L of assay buffer containing the tracer (5 nmol/L) and 20 \(\mu\)L of diluted (1:500–1:1000) ascites fluid are added and mixed. After a 30-min incubation (20 °C), the fluorescence of the strips is measured in a time-resolved fluorometer (Model 1230 Arcus; Wallac Oy).

**Results**

Table 1 gives the fluorescence properties of the tracer used. The quenching caused by antibodies was more pronounced in the albumin-containing buffer and was further optimized by the use of a slightly acidic assay buffer. In the assay buffer described, the maximal quenching caused by anti-thyroxin antibodies was about 90% (Figure 1).

The standard curve for the clinical range of T\(_4\) concentrations (from 10 to 300 nmol/L) (Figure 2) corresponded to the steepest portion of the titration curve (Figure 1).

We assessed the precision of the assay by analyzing 12 replicates of serum-based standards. The resulting precision profile is presented in Figure 2. We also tested the assay by analyzing 30 serum samples that were previously analyzed with commercial RIA. The slope of the regression line was 1.035, the y-intercept was 1.84 nmol/L, and the linear correlation coefficient was 0.967 (Figure 3).

**Table 1. Fluorescence Properties of Eu-Labeled Thyroxin**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Excitation max, nm</th>
<th>Decay time, (\mu)s</th>
<th>Rel. fluorescence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer, pH 7.5</td>
<td>334</td>
<td>270</td>
<td>0.08</td>
</tr>
<tr>
<td>Tris buffer + albumin, 10 g/L</td>
<td>355</td>
<td>428</td>
<td>1.5</td>
</tr>
<tr>
<td>Enhancement solution</td>
<td>340</td>
<td>505</td>
<td>100</td>
</tr>
</tbody>
</table>

*Emission maximum for all experiments, 613 nm.

![Fig. 1. Antibody titration curves for two ascites fluids containing anti-thyroxin](image1)

The amount of tracer was 5 nmol/L. Serial dilutions were made with the assay buffer.

![Fig. 2. A typical standard curve (●) and precision profile (○) for the present thyroxin assay](image2)

**Discussion**

Ordinarily, the fluorescent lanthanide chelates are very sensitive to the environment, especially to water, which, when bound to the free coordination spheres of metals, is an efficient quenching agent (11). For fluorescence of the chelates to be greatest, the chelated lanthanides must be efficiently protected from dissociation and quenching processes. This can be accomplished by using, e.g., detergent micelles and synergistic agents (1), polymeric latex particles (9), or polydentate, stable or inert chelates (8).

By choosing the light-absorbing moiety in such a way that it contains suitable environment-sensitive groups, even highly fluorescent chelates can be used for homogeneous assays. The achieved change can be in the excitation or emission wavelengths, quantum yield, decay time, or degree of polarization.

The chelate we used in this study contained a stable complexing moiety, a functional group (with a spacer arm) for conjugation reactions, and an excitation-light-absorbing aromatic structure in which the excitation and energy-donating properties are sensitive to changes in the chelate environment taking place upon immunoreaction. Free in solution, the tracer is only slightly fluorescent, and the addition of antibodies has a negligible effect on that fluorescence. The addition of detergents or albumin to the assay buffer increases the fluorescence of the free fraction as...
compared with that of the bound. "Quenching" of that fluorescence on alternative binding to either albumin or to antibodies is used here to evaluate the homogeneous assay principles in time-resolved FIA.

Although the fluorescence of the tracer in assay buffer is less intense than the Eu$^{3+}$ fluorescence in the fluorescence enhancement solution (Table 1), it suffices for the thyroxin assay. The considerably large amount of antibody used, sufficient for binding most of the tracer at the zero concentration standard, allows rapid equilibrium in the solution after mixing of the components. This also makes it possible to use pre-equilibrated antibody–tracer solution for a single-reagent system.

Because no separation, washings, and enhancement steps are required, a signal reproducibility of around 1% can be achieved, which, within a standard curve of (e.g.) 2.25-factor signal difference (Figure 2), gives an acceptable precision within the clinical range of thyroxin concentrations, from 50 to 300 nmol/L.

Because constituents of serum do not cause fluorescence background interferences in time-resolved fluorometry (12), sample pretreatment is less important than in conventional homogeneous FIA. Only samples causing exceptionally high absorption of excitation light or samples interfering with the immunoreaction are likely to cause problems for homogeneous time-resolved FIA. In fact, a preliminary study with clinical serum samples showed quite good correlation with RIA (Figure 3). Work continues on further evaluating the utility of the chelate for use in rapid homogeneous assays of haptenic molecules that do not require the most sensitive assay format, e.g., for assays of steroid glucuronides in urine.

REFERENCES

CLIN. CHEM. 34/11, 2322–2327 (1988)

Dual-Precipitation Method Evaluated for Determination of High-Density Lipoprotein (HDL), HDL$_2$, and HDL$_3$ Cholesterol Concentrations

Valerian C. Dias, Howard G. Parsons, Nigel D. Boyd, and Paul Keane

We evaluated a dual-precipitation method for determining cholesterol in high-density lipoprotein (HDL) and its subfractions HDL$_2$ and HDL$_3$. After total HDL was isolated by precipitation of very-low-density (VLDL) and low-density (LDL) lipoproteins with polyethylene glycol (PEG 8000), HDL$_2$ was isolated from total HDL by precipitation with dextran sulfate (M, 15 000), leaving HDL$_3$ in the supernate. Concentration of total HDL cholesterol after precipitation of VLDL and LDL with PEG showed significant proportional and constant biases of −3.8% and 0.04 mmol/L, respectively, when compared with a phosphotungstic acid-based comparison method, although results by the two methods were correlated highly ($r = 0.99$, $P < 0.001$). HDL$_2$ and HDL$_3$ cholesterol concentrations measured with the present technique were not different from those obtained by density-gradient ultracentrifugation or by combined precipitation–ultracentrifugation.

ADDITIONAL KEYPHRASE: precipitation with polyethylene glycol–dextran sulfate–Mg$^{2+}$ and with phosphotungstic acid, and ultracentrifugation methods compared

The negative correlation established between the concentration of high-density lipoprotein (HDL) cholesterol in serum and the risk of coronary artery disease has resulted in an increased demand for assay of this analyte (1, 2). Recently, individual HDL cholesterol subclasses, in particular HDL$_2$, were shown to be a strong negative predictor for coronary heart disease (3, 4).

1 Departments of Pediatrics and 2 Pathology, University of Calga-
ry, 3330 Hospital Drive N.W., Calgary, Alberta, T2N 4N1, Canada.
3 Department of Laboratory Medicine, Foothills Provincial Hospi-
tal, Calgary, Alberta, Canada.
4 Address correspondence to this author.
Received April 10, 1988; accepted July 12, 1988.