Simultaneous Quadruple-Label Fluorometric Immunoassay of Thyroid-Stimulating Hormone, 17α-Hydroxyprogesterone, Immunoreactive Trypsin, and Creatine Kinase MM Isoenzyme in Dried Blood Spots

Yong-Yuan Xu,1,3 Kim Pettersson,2 Kaj Blomberg,2 Ilkka Hemmilä,2,4 Heikki Mikola,2 and Timo Lövgren1

We describe a quadruple-label fluorometric immunoassay for simultaneously measuring four analytes: thyroid-stimulating hormone (TSH), 17α-hydroxyprogesterone (17α-OHP), immunoreactive trypsin (IRT), and creatine kinase MM (CK-MM). The assay is based on immunoreagents labeled with four different lanthanide ions (Eu3+, Tb3+, Sm3+, and Dy3+), respectively; 17α-OHP was labeled with Tb3+. The assay was performed in microtitration strip wells coated with a mixture of monoclonal antibodies against β-TSH, IRT, and CK-MM and a polyclonal goat anti-rabbit IgG for capture of the rabbit anti-17α-OHP antibodies. After completion of the immunoreactions, the bound fractions of the lanthanides were dissociated into the co-fluorescence enhancement solution, creating highly fluorescent chelates. The four lanthanide-specific signals were subsequently measured in a time-resolved fluorometer. The detection limits of the assay were 0.1 mIU/L for TSH, 2 nmol/L for 17α-OHP, 2 µg/L for IRT, and 4 U/L for CK-MM.

Additional Keyphrases: monoclonal antibodies - lanthanide chelates - multiple (“co”) fluorescence - multicomponent analysis - screening - neoplasms

Numerous immunoassay techniques have been developed to screen blood spotted onto filter paper for congenital hypothyroidism by measuring thyroid-stimulating hormone (TSH)1-3, for cystic fibrosis by measuring immunoreactive trypsin (IRT)4,5, and for adrenal hyperplasia by measuring 17α-hydroxyprogesterone (17α-OHP)6-9. Bioluminescent10 and fluorometric11 assays based on the measurement of creatine kinase (CK; EC 2.7.3.2) have been used to screen for Duchenne and Becker muscular dystrophy12. At present, the screening assays are performed as discrete tests, measuring only one analyte at a time. If several analytes are to be measured from a single blood spot, the sample must be divided into fractions, each of which is analyzed separately. However, combining several analyses into a multianalyte immunoassay would save time, labor, and reagents and also reduce the amount of sample needed.

Various assay configurations have been described for multianalyte assays13. One approach is to use multiple sites for different assays, as in the multimicrospot multianalyte assay proposed by Ekins et al.14. Kakabekos et al.15 recently demonstrated a similar approach, using separate "macroscopic" areas and Eu chelate labels to combine temporal and spatial resolutions in the fluorometric detection. Triple- and quadruple-label immunoassays have not, so far, been realized in the field of quantitative immunoassays. The problems in designing triple- or quadruple-label assays relate to the difficulties in finding three or four labels easily distinguishable from each other to allow the combination of highly sensitive, broad-range assays of both competitive and noncompetitive design. The combination of temporal and spectral resolution in time-resolved fluorometry makes it possible to considerably increase the specificity of signal detection and thus gives an opportunity to develop multilabel technologies, especially when measuring the nonoverlapping emissions of various long decays created by the lanthanide chelates.

Dual-label assays have been used for determining various analyte pairs16. Eu3+ and Tb3+ have been used in dual-label time-resolved immunofluorometric assays of lutropin (luteinizing hormone) and follitropin (follicle-stimulating hormone)17 and of adeno- and rotaviruses18. Eu3+ and Sm3+ is the other label pair combined in dual-label assays, e.g., in the detection of plant viruses19 and in screening for Down syndrome2. All these assays were based on the principle of dissociative fluorescence enhancement: after completion of the immunoreaction, an enhancement solution was added to dissociate the lanthanide ions and to enhance the

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5 Nonstandard abbreviations: TSH, thyroid-stimulating hormone (thyrotropin); 17α-OHP, 17α-hydroxyprogesterone; IRT, immunoreactive trypsin; CK-MM, creatine kinase MM; CFES, co-fluorescence enhancement solution; β-NTA, β-naphthyltrifluoroacetone; and PTA, pivaloyltrifluoroacetone.

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amount of fluorescence they produced. However, these direct enhancement solutions, based on either β-naphthyltrifluoracetone (β-NTA) or pivaloyl trifluoracetone (PTA) (16) as the enhancing ligands, cannot be used for the simultaneous determination of more than two labels at a time with the sensitivity required.

We have developed several co-fluorescence-based enhancement solutions (CFES) (20), some of which are well suited for the simultaneous measurement of four lanthanide ions—Eu³⁺, Tb³⁺, Sm³⁺, and Dy³⁺—with high sensitivities (21, 22). Here, we describe an application of the PTA-yttrium-based CFES (21) in a quadruple-label, time-resolved fluorometric immunoassay for measuring TSH, 17α-OHP, IRT, and CK-MM from dried blood samples spotted onto filter paper.

Materials and Methods

Instruments

We used a Model LS 5 Luminescence Spectrometer (Perkin-Elmer Co., Norwalk, CT) in the phosphorescence mode for the spectral analysis of the chelate fluorences. The fluorometric determination of the lanthanide ions and the calculation of results were performed with a Model 1230 Arcus time-resolved fluorometer (Wallac Oy, Turku, Finland) equipped with narrow-band interference filters for Eu³⁺ (613 nm), Tb³⁺ (545 nm), Sm³⁺ (644 nm), and Dy³⁺ (573 nm) and a wide-band excitation filter (250–400 nm). Table 1 presents the instrumental measurement conditions used for the different ions.

Reagents

PTA was synthesized by Claisen condensation reaction from pivaloylmethane and the ethyl ester of trifluoracetate, with NaH as the condensing agent (23). Yttrium oxide (99.999%) was obtained from Alfa, Karlsruhe, Germany; 1,10-phenanthroline (99%) from Aldrich Chemie, Steinheim, Germany; and Triton X-100 from Fluka, Buchs, Switzerland. Absolute ethanol was supplied by Alko, Helsinki, Finland. 2,4,17α-Pregnadien-20-yno(2,3-dijisosoxazol-17-ol (danazol) was purchased from Sigma Chemical Co., St. Louis, MO. The 3-(O-carboxymethyl)oxime of 17α-OHP was synthesized according to the method of Janoeki et al. (24).

For the two-site immunometric assays of TSH and IRT, we used the monoclonal antibodies of the respective Delfia neonatal screening kits (Wallac). To assay CK-MM, we used a monoclonal antibody against CK-MM from OEM Concepts (Toms River, NJ) as a capture antibody and a polyclonal goat anti-CK-MM antibody from BioPacific (Emeryville, CA) as the labeled reagent. The polyclonal goat anti-rabbit IgG was obtained from Dako, Denmark; rabbit anti-17α-OHP was from Wallac, as were dried blood-spot standards for TSH, liquid standards for IRT and 17α-OHP, Delfia Wash Solution, and Eu³⁺- and Sm³⁺-labeling reagents. Labelings with Tb³⁺ and Dy³⁺ were performed with the Tb³⁺ and Dy³⁺ chelates of N₁-(p-aminobenzyl)- and N₁-(p-isothiocyanatobenzyl-) derivatives of diethylentriamine-N₁,N₂,N₃,N₃-tetraacetic acids, synthesized at Wallac according to the method of Mukkala et al. (25). Highly purified CK-MM standard was obtained from Aalto Scientific (Fallbrook, CA). The assay buffer contained 9 g of NaCl, 0.5 g of NaK₃, 10 g of bovine serum albumin, 0.5 g of bovine globulin, 0.1 mL of Tween 40, 20 μmol of diethylenetriaminopentaacetic acid, 5 g of polyethylene glycol, and 0.3 mg of danazol per liter of 50 mmol/L Tris · HCl buffer, pH 7.75.

Co-Fluorescence Enhancement Solution

The CFES based on PTA-Y³⁺ was as described elsewhere (21). The CFES comprises two parts: the dissociative part, 70 μmol of PTA, 7.5 μmol of Y³⁺, 0.6 g of Triton X-100, and 300 mL of ethanol per liter, adjusted to pH 5.5 with acetic acid; and the enhancing part, 0.5 mmol of phenanthroline and 0.2 mol of Tris per liter. Stored at 4 °C in the dark, the CFES was brought to room temperature just before use.

Standards

To simplify the standardization of the quadruple-label assay, we arranged the standards to give increasing ion concentrations. Thus the standard concentrations for the three noncompetitive analyses were arranged in ascending order but for the competitive 17α-OHP assay in descending order (Table 2). This arrangement avoided the need to correct for signal cross-talk between the signals for the standards (for unknown samples, this correction still had to be made).

Procedures

Labeling the immunoreagents. The labeling of anti-TSH, anti-IRT, and goat-anti-CK-MM with Eu³⁺, Sm³⁺, and Dy³⁺, respectively, was performed in overnight incubations, with 50- to 500-fold molar excesses of the labeling reagents in a 50 mmol/L carbonate buffer, pH 8.5 or 9.8, at 4 °C or at room temperature. The labeled

<table>
<thead>
<tr>
<th>Fluorescent Ion</th>
<th>Cycling time</th>
<th>Delay time</th>
<th>Counting time</th>
<th>Emission filter, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eu³⁺</td>
<td>2</td>
<td>0.5</td>
<td>1.5</td>
<td>613</td>
</tr>
<tr>
<td>Tb³⁺</td>
<td>1</td>
<td>0.4</td>
<td>0.5</td>
<td>545</td>
</tr>
<tr>
<td>Sm³⁺</td>
<td>1</td>
<td>0.05</td>
<td>0.2</td>
<td>644</td>
</tr>
<tr>
<td>Dy³⁺</td>
<td>1</td>
<td>0.05</td>
<td>0.1</td>
<td>573</td>
</tr>
</tbody>
</table>

Table 2. Combination of the Four Standards in the Quadruple-Label Assay

<table>
<thead>
<tr>
<th>Standard</th>
<th>Combined standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
</tr>
</tbody>
</table>

| TSH, mIU/L | 1     |
| 17α-OHP, nmol/L | 100   |
| IRT, μg/L   | 25    |
| CK-MM, U/L  | 10    |

Table 1. Time-Resolved Fluorometric Measuring Conditions with CFES

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antibodies were separated from the free chelates and possible protein aggregates by gel filtration on a combined column of Sephadex G-50 and Sepharose 6B (Pharmacia, Uppsala, Sweden). The incorporation yields for the TSH, IRT, and CK-MM antibodies were 10 Eu³⁺, 15 Sm³⁺, and 20 Dy³⁺ ions per molecule of IgG, respectively. The 3-(O-carboxymethyl)oxime of 17α-OHP was conjugated with the p-aminobenzyl derivative of the Tb³⁺ chelate by a modified procedure developed for labeling carboxy-derivatized steroids (26).

Coating. Microtitration strip wells (Eflab, Helsinki, Finland) were coated with a mixture of monoclonal anti-β-TSH, anti-IRT, and anti-CK-MM antibodies and polyclonal goat anti-rabbit IgG, 0.7 μg of each antibody per well, at 37 °C in overnight incubation at pH 4.5. The coated strips were blocked and stabilized by incubation with bovine serum albumin, 1 g/L, then aspirated and stored wet at 4 °C.

Quadruple-label time-resolved fluorometric immunoassay. The immunoassay was performed in the coated microtitration strip wells. The immunoreactions were carried out in the presence of a dried blood-spot standard for TSH (5 mm diameter); 25 μL of combined liquid standards for 17α-OHP, IRT, and CK-MM; 50 ng of Eu-anti-TSH, 100 ng of Sm-anti-IRT, 300 ng of Dy-anti-CK-MM, and 0.09 pmol of Tb-labeled 17α-OHP; and a 75,000-fold dilution of rabbit anti-17α-OHP antiserum in 200 μL of assay buffer. The incubation was performed overnight at 4 °C. After six washings, the bound fractions of Eu³⁺, Tb³⁺, Sm³⁺, and Dy³⁺ were dissociated by shaking for 4 min in 200 μL of the dissociative part of the CFES. The fluorences of the labels were enhanced by adding 20 μL of the enhancement part of the CFES and shaking for 7 or 10 min. The mixture was allowed to stand for an additional 10 min, after which the fluorescence intensities of each ion were measured in the time-resolved fluorometer according to the measurement conditions specified in Table 1.

Sample Specimens

Dried blood specimens were kindly donated by Dr. Nørgaard-Pedersen (Statens Seruminstitut, Copenhagen, Denmark), Dr. Scheuerbrandt (CK Test Laboratorium, Breitnau, FRG), and Dr. Torresani (Kinderspital, Zürich, Switzerland).

Results

Simultaneous Detection of Eu³⁺, Tb³⁺, Sm³⁺, and Dy³⁺

A three-dimensional profile, including the emission intensity, emission wavelength, and time delay, of the lanthanide-chelate luminescences in the CFES after excitation at 315 nm is shown in Figure 1. The strongest emission peaks were obtained at 612 nm for Eu³⁺, 544 nm for Tb³⁺, 647 nm for Sm³⁺, and 574 nm for Dy³⁺; the respective decay times were 820, 323, 88, and 27 μs. Table 3 presents the results of a time-resolved fluorescence measurement of the four ions. The detection limit obtained with Eu³⁺ is sufficient to allow measurement of antigen amounts as little as 1–10 amol (10⁻¹⁸ to 10⁻¹⁷ mol) per test, whereas that of Dy³⁺, because of its lower signal and its short decay time, enables only applications requiring a moderate or low sensitivity [1–10 fmol (10⁻¹⁴ to 10⁻¹⁵ mol) per test]. The spectral cross-talk factors in the fluorometry of the ions are given in Table 4.

Quadruple-Label Assay

Figure 2 shows the dose–response curves of the assay and the precision profiles calculated from 12 replicates of the standards. The detection limits were 0.1 mIU/L for TSH, 2 nmol/L for 17α-OHP, 2 μg/L for IRT, and 4 U/L for CK-MM, as calculated from the imprecision of the lowest standard (1 mIU/L standard for TSH and zero standard for others) and defined as the response giving a signal equivalent to the mean ± 3 SD of the signal for the zero standard.

Table 5 lists the detection limits, dynamic ranges, and precisions of the assays of TSH, 17α-OHP, and IRT of the quadruple-label assay, in comparison with the respective Delfia single-label assays. The detection limit of the co-fluorescent TSH assay is 10-fold lower than that of the corresponding Delfia kits, whereas the re-
verse is true for IRT (reflecting the detection sensitivities of the respective labels). For 17α-OHP, there are no differences in detection limits between the two assay types. The detection limits fulfill well the requirements for a screening assay with respect to the clinical cutoff values used in routine neonatal screening: 20–25 mIU/L for TSH, 30–60 nmol/L for 17α-OHP, and ~70 μg/L for IRT. The detection limit of the quadruple-label assay for CK-MM (4 U/L) is also well below the commonly used cutoff value, 180–500 U/L, estimated with an enzymatic measurement of CK activity (10). Regardless of the high concentrations present, no high-dose hook effect was observed, even with samples containing CK-MM up to 5000–10 000 U/L.

We further evaluated the present method by measuring 56 neonatal blood-spot samples. The mean (SD) values obtained were 2.9 (4.1) mIU/L, 17.4 (12.3) μg/L, and 28.5 (5.9) nmol/L for TSH, IRT, and 17α-OHP, respectively. These values agreed well with the values obtained with Delfia kits. The mean value for CK-MM was 38.1 (SD 38) U/L. Ten samples from neonates with confirmed congenital hypothyroidism were detected equally well with the quadruple-label assay of TSH (y) as with the single-label Delfia (x): y = 1.08 x – 18.1 mIU/L. In three confirmed cases of congenital adrenal hyperplasia, the measured 17α-OHP concentrations were above the measurement range (>250 nmol/L) with both assays. No samples from positive cases of cystic fibrosis were available. CK-MM concentrations in positive muscular dystrophy samples, predetermined with an enzymatic test (10) to have CK-MM concentrations >4000 and >5000 U/L, were also well above the highest concentration standard used in the quadruple-label assay.

Discussion

Two principles have been suggested for multianalyte assays: use of multiple labels or multiple space techniques (13). In both approaches the fluorometric detection principle, particularly time-resolved fluorometry, has played an important role in creating the required label detection sensitivity and specificity (14, 15).

Using a single time-resolved fluorometric detection system and a dissociative fluorescence enhancement, one can detect as many as four fluorescent labels simultaneously. In the earlier published double-label assays, direct enhancement systems, in which the chelates formed had the structure Ln3+ (β-diketone)3(tri-n-octylphosphine oxide)2–, detected Eu3+ (27) and Sm3+ (28) with aromatic β-diketones (e.g., β-NTA), and detected Tb3+ and Eu3+ (17, 28) with aliphatic β-diketones (e.g., PTA). The detection limits of the β-NTA-based enhancement solution (Delfia enhancement solution) are 0.039 pmol/L for Eu3+ and 3.3 pmol/L for Sm3+. The PTA-based direct enhancement solution could detect as little Eu3+ and Tb3+ as 1 and 40 pmol/L, respectively (29). Even though it is theoretically possi-

Table 5. Quadruple-Label Assay and Corresponding Single-Label Assays Compared for Three Analytes

<table>
<thead>
<tr>
<th>Quadruple-label assay</th>
<th>Delfia</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH, 17-OHP, IRT, CK-MM</td>
<td></td>
</tr>
<tr>
<td>mIU/L, nmol/L, μg/L</td>
<td>mIU/L, nmol/L, μg/L</td>
</tr>
<tr>
<td>Assay range</td>
<td>1–250 0–250 0–1000</td>
</tr>
<tr>
<td>Detection limit</td>
<td>0.1 2 2</td>
</tr>
<tr>
<td>Precision, %</td>
<td>3–5 2–10 2–8</td>
</tr>
</tbody>
</table>

Fig. 2. Dose–response curves (●) and precision profiles (▼) of TSH, 17α-OHP, IRT, and CK-MM measured with quadruple-label immunoassay.
able to measure all four lanthanides with the PTA-based direct enhancement solution, the detection limits achieved for Sm\(^{3+}\) and Dy\(^{3+}\) are seriously limited by their lower quantum yields, lower excitation maxima, and in particular, shorter decay times. The use of intermolecular energy transfer to amplify the excitation efficiency in CFES enables the increase in both the fluorescence intensities and in the decay times with all the ions (Table 3) (21) and yields practicable detection limits also for Sm\(^{3+}\) and Dy\(^{3+}\). A very important factor affecting the detection sensitivity is the fluorescence background, which depends both on the wavelengths used and on the delay times used. The co-fluorescence effect prolonged the decay times of all the fluorescent ions, especially that of Dy\(^{3+}\) (from ~1 to 27 \(\mu\)s), which strongly decreased the measured background.

Another problem relating to multianalyte immunoassay arises from the cross-talk between the signals originating from different labels. However, because of the considerable differences not only in the emission spectra but also in the decay times of the four ions in CFES (Table 1), the fluorescence interferences were negligible or insignificant except for that from Sm\(^{3+}\) to Dy\(^{3+}\) (Table 3). A simple mathematical correction can be used after measuring all the ions to subtract the unwanted spillover signal when needed.

In designing the quadruple-label assay, we assigned the labels according to the sensitivity required for each analyte, the fluorescence background of CFES, and the fluorescence interference. Because the assays of TSH and 17\(\alpha\)-OHP required greater sensitivities (lower detection limits), Eu\(^{3+}\) and Tb\(^{3+}\) were assigned to these analytes. Because the interference from Sm\(^{3+}\) in the detection of Dy\(^{3+}\) was considerable and because the amount of fluorescence produced by Dy\(^{3+}\) exceeded that of Sm\(^{3+}\) (Table 3), we assigned Dy\(^{3+}\) to the CK-MM assay, which gives a greater signal than the IRT assay. In addition, by controlling the amount of label, the signals can be adjusted to decrease the possible spillover problem; therefore, we used a higher concentration of label for Dy\(^{3+}\) (20 Dy\(^{3+}\) per IgM molecule) than for Sm\(^{3+}\) (15 Sm\(^{3+}\) per IgG molecule). In the assay of unknown samples, the raw data were recalculated according to the factors in Table 4 to obtain corrected results.

Despite the accommodation of four solid-phase assays in the same well sharing the same limited binding capacity of the microtitration well surface, the dynamic ranges were similar to those of the respective single-label assays. This is partly explained by the fact that the sample volume used in the test, ~4 \(\mu\)L of plasma, is relatively small; therefore, antibody excess situations are easily achieved in ratio to the analyte concentration likely to be found in this volume of sample. This is also illustrated by the fact that the dynamic range of the single-label Delfia TSH kit, which requires 50-\(\mu\)L serum samples, is >10-fold the range needed in neonatal screening. In addition, we used more antibody to coat the microtitration strip wells in the present study than is used in the single-label Delfia kits, for a more efficient utilization of the available surface.

The changes in detection limits for the TSH and IRT measurements in the present study compared with those for the single-label Delfia kits are likely to have no practical consequences in routine screening. IRT, being less sensitive in the quadruple-label assay, still has a detection limit (2 \(\mu\)g/L) well below the commonly used cutoff value for cystic fibrosis (~70 \(\mu\)g/L). On the other hand, the improved sensitivity of the TSH assay is still not sufficient for reliable detection of secondary congenital hypothyroidism (30). The sensitivity of the CK-MM determination, based on use of the least-sensitive lanthanide label, Dy\(^{3+}\), is more than adequate for detecting muscular dystrophy, considering the current cutoff values of 180-500 U/L (10). On the other hand, CK-MM values can be very high in children with Duchenne/Becker muscular dystrophy, especially beyond the neonatal period. Our preliminary experiments with CK-MM concentrations as great as 10 000 U/L showed no danger of obtaining artifactually negative results from a high-dose hook effect.

The results of the quadruple-label assay from an admittedly limited number of normal neonatal samples and positive samples from several cases of congenital hypothyroidism, congenital adrenal hyperplasia, and muscular dystrophy showed good agreement with the results of the corresponding single-label tests and a reassuringly good resolution of the positive cases. For further validation of the method, additional data are needed over extended periods under routine screening conditions.

References
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