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ENHANCED LUMINESCENT ENZYME IMMUNOASSAYS FOR RUBELLA ANTIBODY, IMMUNOGLOBULIN E AND DIGOXIN

Gary H G Thorpe, Robert Haggart,
Larry J Kricka,* and Thomas P Whitehead

Department of Clinical Chemistry,
Wolfson Research Laboratories,
Queen Elizabeth Medical Centre
and
* University of Birmingham,
Birmingham B15 2TH, UK

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SUMMARY: A novel firefly luciferin-enhanced luminescent procedure for the quantitation of horseradish peroxidase labels has been directly incorporated into established enzyme immunoassays. The procedure is rapid and sensitive and uses readily available reagents. Light emission from the enhanced reaction is high and relatively constant and thus easily measured. The luminescence procedure has been successfully incorporated into immunometric assays for rubella antibody and human IgE and into a competitive immunoassay for digoxin.

Light emission from the horseradish peroxidase-catalysed oxidation of luminol, isoluminol or 7-dimethylaminonaphthalene-1,2-dicarbonic acid hydrazide is enhanced by synthetic firefly D-luciferin (4,5-dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazole-carboxylic acid) (1-2). Horseradish peroxidase (EC 1.11.1.7) is widely used as a label in enzyme immunoassay and can be assayed by several bio- or chemiluminescent procedures (3-8). The low light intensity, rapid decay of light emission, interference or limited reagent availability may, however, limit their applicability. The major advantages of the new luciferin-enhanced luminescent procedure are that the light levels are relatively high and thus more readily detectable, and the light emission decays slowly (the reaction mixtures glowing for several

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minutes). In addition the assay is rapid and sensitive and employs readily available reagents.

In this paper we describe the direct incorporation of the luciferin-enhanced luminescent quantitation of horseradish peroxidase conjugates into existing immunoassays. The assays chosen are representative of immunometric and competitive assays and employ two widely used solid supports; plastic beads and tubes.

MATERIALS AND METHODS

Reagents Firefly D-luciferin, luminol, Tris-HCl buffer, and hydrogen peroxide (30% w/v) were purchased from Sigma Chemical Company. Luminol was purified by conversion to its sodium salt as described previously (9).

Light measurement This was performed in a luminometer based on a side-window photomultiplier tube (EMI type 9781A, 94 μA/lumen) and using photocurrent measurements (10).

Colorimetry All measurements were performed on a Shimadzu UV-240 recording spectrophotometer.

Clinical specimens A series of 34 specimens with a range of rubella antibody concentrations were kindly supplied by the Regional Virology Laboratory, East Birmingham Hospital, Birmingham.

Assay for anti-rubella virus IgG Assays were carried out in duplicate using a Rubazyme kit (Abbott Diagnostics Division, Hampshire, UK). The assay involves the incubation of a rubella virus coated polystyrene bead first with the sample and then with horseradish peroxidase labelled anti-human IgG antibody for 1 h at 37°C. After the final washing, bound conjugate was quantitated using a colorimetric or enhanced luminescent end-point.

Colorimetric end-point Beads were incubated in the dark with a mixture of ortho-phenylenediamine (OPD) - hydrogen peroxide for 0.5 h at room temperature. The reaction was terminated using hydrochloric acid (1 M) and the absorbance read at 492 nm.

Enhanced luminescence end-point Beads were transferred into disposable plastic cuvettes (W Sarstedt (UK) Ltd, Leicester, UK, 1 x 1 x 4.5 cm) containing 0.5 ml Tris buffer (0.01 M, pH 8.0). The enhanced luminescent reaction was initiated by manual addition of 10 μl firefly D-luciferin (3.6 mM in the Tris buffer) and 0.5 ml of a mixture of luminol (2.5 mM) and hydrogen peroxide (5.4 mM) in the Tris buffer. Any bubbles adhering to the surface of the beads were removed by gently shaking the cuvette which was then placed in the luminometer. The intensity of light emission after 30 seconds was recorded. The geometry of the luminometer was such that beads always occupied the same position relative to the photomultiplier tube.

Assay for human IgE An Enzygnost kit (Hoechst UK Ltd, Middlesex, UK) was used to assay a series of standards and the control supplied with the kit. The assay used plastic tubes coated with anti-IgE which were incubated successively with a diluted serum sample and horseradish peroxidase labelled anti-human IgE for 2 h at room temperature.

Colorimetric end-point Tubes were incubated with a mixture of OPD-hydrogen peroxide in the dark for 0.5 h at room temperature, the reaction was stopped by addition of sulphuric acid (0.5 M), and the absorbance at 492 nm was measured.

Enhanced luminescence end-point Tubes were aspirated to dryness and light emission was initiated by manual addition 10 µl firefly D-luciferin (3.6 mM) in Tris buffer (0.01 M, pH 8.0) and 1.0 ml of a mixture of luminol (1.25 mM) and hydrogen peroxide (2.7 mM) in the Tris buffer. The tube was then placed in the luminometer and the intensity of light emission after 30 seconds was recorded.

Assay for serum digoxin An Enzymun-Test Digoxin kit (BCL Ltd, East Sussex, UK) was used to assay standards and control supplied with the kit. The assay involves incubation of tubes coated with anti-digoxin with a mixture of the sample and horseradish peroxidase labelled digoxin for 45 minutes at room temperature.

Colorimetric end-point Tubes were incubated in the dark with the 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonate)-perborate substrate for 1 h at room temperature. Tube contents were transferred to cuvettes and the absorbance was measured at 420 nm.

Enhanced luminescence end-point Tubes were aspirated to dryness and 0.6 ml Tris buffer (0.01 M, pH 8.0) was added to each tube. The luminescent reaction was initiated by adding 10 µl firefly D-luciferin (3.6 mM) in the Tris buffer and 0.4 ml of a mixture of luminol (3.1 mM) and hydrogen peroxide (7.3 mM) in the Tris buffer. The intensity of light emission after 30 seconds was then measured.

RESULTS

Rubella antibody assay Figure 1 illustrates the kinetics of the luciferin-enhanced luminescent emission from beads. For convenience a time of 30 seconds was chosen for the measurement of light intensity, but as can be seen light emission is prolonged and the measurements could be made either earlier or several minutes later. Similar discrimination between the three controls was obtained using assays with an enhanced luminescent and a colorimetric end-point. For a series of 34 specimens, good correlation ($r = 0.97$, regression line $y = 0.94x + 0.18$) was

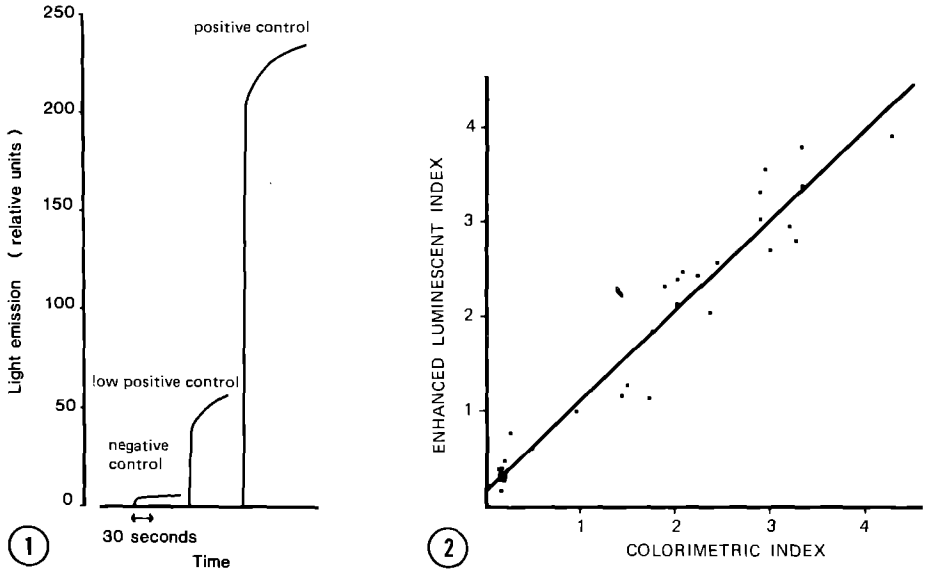

Figure 1 Kinetics of light emission using the firefly luciferin-enhanced luminescent reaction in an immunometric assay for rubella antibody employing beads as solid support. Assays were performed as described in Materials and Methods.

Figure 2 Comparison of serum rubella antibody levels using an immunometric assay with either an enhanced luminescent or a colorimetric end-point. The index for each specimen was obtained by dividing its response by that of the low positive control.

obtained between results of the colorimetric and enhanced luminescent rubella antibody assays (Figure 2).

IgE and digoxin assays The standard curves for each assay obtained using the colorimetric and enhanced luminescent end-points are shown in Figures 3 and 4. For the two assays the standard curves were similar and appropriate values for controls were obtained. In the digoxin assay use of the enhanced luminescent end-point halved the overall assay time without any loss in sensitivity (Figure 4), despite the use of tubes not purpose-designed for luminescent monitoring and the fact that only 50% of their active surface was visible to the photodetector.

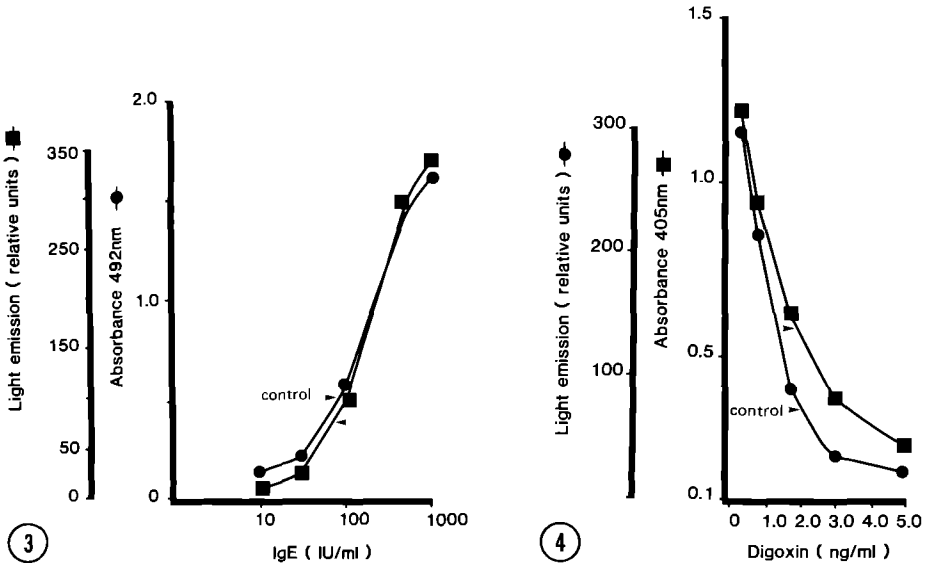

Figure 3 Standard curves of human IgE in an immunometric assay using tubes as solid supports and quantitation of peroxidase labels with either an enhanced luminescent or a colorimetric end-point. Values for control sera are indicated.

Figure 4 Standard curves of digoxin in an immunoassay using tubes as solid support and quantitation with either an enhanced luminescent or a colorimetric end-point. Values for control sera are indicated.

DISCUSSION

There is a growing interest in the use of luminescent reactions to monitor immunoassays (11-13). The reaction between horseradish peroxidase, oxidant and cyclic diacylhydrazides has been used to quantitate horseradish peroxidase labels (4-8) but it exhibits rapid kinetics and is prone to interference at high pH, while at near neutral pH the light emission is of a low intensity. Synthetic firefly luciferin enhances light emission from the commonly employed cyclic diacylhydrazides (luminol, isoluminol and 7-dimethylaminonaphthelene-1,2-dicarbonic acid hydrazide) and we have successfully incorporated this novel luminescent end-point into a series of existing immunoassays based on horseradish peroxidase conjugates. The main advantages of the firefly luciferin-enhanced luminol-peroxide assay for

horseradish peroxidase are that it is rapid, light emission is relatively constant over many minutes, and sensitivity is improved compared with the assay without luciferin. The enhanced luminescent assay does not require the lengthy temperature-controlled incubations used in colorimetric procedures and thus simplifies and greatly shortens the overall immunoassay time.

The relatively constant light emission eliminates any requirement for rapid reproducible mixing in front of the photodetector and both the signal:background ratio of the luminescent reaction and level of light emission ensure good sensitivity using simple instrumentation. It is expected that further improvements in sensitivity would be obtained if the assay was reoptimised using instrumentation with a more sensitive photodetector (such as a cooled end-window photomultiplier tube) and if more of the light emitted were collected by reflectors and the light collection system matched with the geometry of the solid support employed. No significant interference from serum components was encountered in any of the heterogeneous immunoassays employed. The enhanced luminescent assay is compatible with a wide range of solid supports and has general applicability to many of the numerous enzyme immmunoassays based on peroxidase labels.

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