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# A new enzyme-linked fluorescence assay (ELFA) for use with peroxidase-antibody conjugates: a comparison with ELISA for the quantitation of IgM antibodies to hepatitis B core antigen

J. R. DAVE, P. TAYLOR, J. M. GRANGE AND H. GAYA

*Departments of Microbiology, Brompton Hospital and Cardiothoracic Institute, Fulham Road, London SW3 6HP*

**Summary.** A new enzyme-linked fluorescence assay (ELFA) suitable for use with peroxidase-antibody conjugates is described. The substrate for the assay is *p*-hydroxyphenylacetic acid, the fluorescent product of which is stable and unaffected by light. The assay compared favourably with a standard ELISA for the quantitation of IgM antibodies to hepatitis B core antigen.

## Introduction

Enzyme-linked immunosorbent assay (ELISA) is widely used as an economical and safe alternative to radioimmunoassay and many test kits are commercially available. In 1979, Yolken and Stopa demonstrated that the sensitivity of ELISA could be significantly increased by substituting a fluorogenic substrate for a chromogenic one and by measuring the resulting fluorescence in a fluorometer. The most widely used substrate is 4-methylumbelliferyl phosphate which is split by alkaline phosphatase to yield 4-methylumbelliferone (4-methyl-7-hydroxycoumarin), one of the most intensely fluorescent substances known (Yolken and Leister, 1982).

In some ELISA systems, antibodies conjugated to horse-radish peroxidase are used and it was therefore considered desirable to find a fluorogenic substrate for use with this enzyme. Guilbault *et al.* (1968) studied 25 compounds that were oxidised to highly fluorescent compounds by the free oxygen radical liberated from hydrogen peroxide by the peroxidase enzyme. The most suitable of these was *p*-hydroxyphenylacetic acid, because it has a high fluorescence co-efficient and is completely stable to auto-oxidation. In this study, the usefulness of this compound as a substrate for an enzyme-linked fluorescence assay (ELFA) based on peroxidase is evaluated and compared with peroxidase ELISA for the quantitation of human IgM antibody to hepatitis B virus core antigen (HBcAg).

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## Materials and methods

### *The principle of the assays*

Wells of microtitration plates were coated with anti-human IgM ($\mu$-chain specific). Samples of serum from patients were then added to the wells so that IgM antibodies were captured; the excess was removed by washing. The hepatitis B virus core antigen (HBcAg) was added and after further washing the amount of HBcAg binding to the IgM antibodies was quantitated by adding enzyme-labelled human anti-HBcAg IgG antibody. The amount of enzyme was then assayed by adding the chromogenic (ELISA) or fluorogenic (ELFA) substrates.

### *Anti-human IgM*

Rabbit anti-human IgM, $\mu$-chain specific (Dako, Copenhagen) and sheep anti-human IgM, $\mu$-chain specific (Seward Laboratories, London) were used at dilutions of 1 in 400 and 1 in 300 respectively, the optimum dilutions determined by chessboard titrations. The antisera were diluted in coating buffer—$Na_2CO_3$ 1·59 g/L and $NaHCO_3$ 2·93 g/L in distilled water, *p*H 9·6.

### *Antigen*

HBcAg derived from genetically modified *Escherichia coli* was kindly donated by Biogen Laboratories, Geneva. On the basis of chessboard titrations, the antigen was used at a dilution of 1 in 10000 in diluent buffer—phosphate-buffered saline (PBS), *p*H 7·2, containing bovine serum albumin fraction V (Armour pharmaceuticals) 5% w/v, sterilised by membrane filtration and stored at −20°C.

### *Test sera*

Sera were obtained from: five patients who were sero-

negative for HBsAg but had IgM antibodies against hepatitis A or Epstein Barr virus; seven HBsAg-negative patients with jaundice of unknown aetiology; 20 uninfected subjects whose sera contained rheumatoid factor; 13 individuals whose sera were negative for HBsAg but gave strong non-specific agglutination in a reverse passive haemagglutination test (Hepatest, Wellcome); 10 carriers of hepatitis B surface antigen (HBsAg); and 11 patients with acute hepatitis B (35 sequential sera). Sera were stored at −70°C and were diluted 1 in 5000 in the diluent buffer (see above) before use.

### *Human anti-HBc IgG antibody*

Serum was obtained from a patient with a titre of anti-HBc antibody $> 10^5$ as determined by the Corzyme kit (Abbot Laboratories). The serum was positive for HBsAg and HBeAg but negative for anti-HBs and anti-HBe when tested by commercially available ELISA kits (Ausab and Abbott-HBe). The IgG fraction was separated by chromatography on DEAE Affi-Gel Blue (Bio-Rad) used according to the manufacturer's instructions. The immunoglobulin content of the eluted fractions was determined by the Beckman ICS Analyser II. Those fractions containing IgG but not IgM nor IgA were pooled and concentrated to a protein content of 8 mg/ml by the addition of Lyphogel (Gelman Sciences Ltd.). The IgG preparation was conjugated to horse-radish peroxidase by the periodate method (Nakane and Kawaoi, 1974) and stored at 4°C. The optimal dilution of the conjugate was found by chessboard titration to be 1 in 700.

### *Wash buffer*

This buffer consisted of PBS, *p*H 7·2, prepared from PBS tablets (Oxoid) with the addition of Tween 20 0·05% v/v.

### *Substrate for ELISA*

The buffer was prepared as two solutions in distilled water—A: citric acid, 21·01 g/L; B: $Na_2HPO_4$. $12H_2O$ 71·6 g/L. Solution A was added to solution B until the *p*H was reduced to 5·0. The substrate solution was freshly prepared by dissolving 34 mg of *o*-phenylenediamine (OPD, Sigma) in 100 ml of buffer. Immediately before use, 5 μl of $H_2O_2$ 3% was added to 10 ml of the substrate solution and the reagent was kept away from direct light.

### *Substrate for ELFA*

The buffer was 0·05M Tris-HCl, *p*H 7·8, prepared by dissolving Tris-hydroxymethyl aminomethane 6·1 g in 50 ml of distilled water, adding 35 ml of N HCl and diluting with distilled water to a final volume of 1L. The substrate solution was freshly prepared by dissolving *p*-hydroxyphenylacetic acid (PHPA, Koch-Light) 37 mg in 10 ml of buffer. Immediately before use, 5 μl of $H_2O_2$ 3% was added.

### *The assay*

The wells of flat bottomed microtitration plates (Dynatech M129A) were coated with 100 μl of diluted anti-human IgM and incubated 4°C for 24 h. The plates were then washed with three changes of wash buffer for 2 min each. A 100-μl volume of diluted test serum was added to duplicate wells, the plate was incubated at 40°C for 1 h and washed three times. A 100-μl volume of diluted antigen was added to each well, the plate was incubated at room temperature for 18–24 h and again washed three times.

For the ELISA, 100 μl of OPD reagent (containing $H_2O_2$) was added to each well and the plate was incubated for 30 min in the dark at room temperature. The reaction was then stopped by adding 50 μl of N HCl. The plates were read on the Intermed Immunoreader NJ-2000 at the dual wavelengths of 490 and 620 nm to minimise errors due to the non-specific absorbance of light.

For the ELFA, the plate was rinsed twice in wash buffer and then once in Tris-HCl buffer and 100 μl of the PHPA reagent (containing $H_2O_2$) was added to each well. The plate was incubated for 30 min at room temperature and 100 μl of Tris-HCl buffer was then added. A 100-μl volume of the reaction mixture was added to 900 μl of Tris-HCl buffer in a cuvette and the flurorescence was read in an Elmer Perkin Spectrofluorometer at excitation and emission wavelengths of 316 and 414 nm respectively.

In each test the results were corrected for those of control sera. The ELISA results were expressed in optical density readings and the ELFA results as arbitrary potentiometer readings. Correlations were calculated by use of Spearman's rank correlation coefficient and the significance of differences in antibody binding in the various groups of sera was determined by the Mann Whitney test.

## Results

Preliminary studies showed that the development of fluorescence from PHPA was unaffected by light and that, once developed, the fluorescence remained stable for at least 24 h.

The table shows the means, medians and ranges of the IgM anti-HBcAg in the five groups of sera when tested by ELISA and ELFA. Low values were found among sera from patients with other viral infections or jaundice of unknown aetiology and from the uninfected rheumatoid factor-positive controls; these were taken together as the control group. The levels of antibody among the HBsAg carriers were significantly higher than those of the control group but significantly lower than those of the patients with acute disease ($p < 0{\cdot}001$ in each case). Two of the 13 sera that gave non-specific agglutination in the Hepatest had levels of IgM anti-HBcAg antibody above the control range but, overall, the antibody levels in this group were not significantly higher than those of the control group.

**Table.** The means, medians and ranges of IgM anti-HBcAg levels in the various groups of sera in the ELISA and ELFA tests

| Group of sera | Assay | Mean* | Standard* deviation | Range* | Median* | Spearman's *r* |
|---|---|---|---|---|---|---|
| Undiagnosed jaundice and other viral infections | ELISA | 0·052 | 0·007 | 0·035–0·064 | 0·050 | 0·54 |
| | ELFA | 0·053 | 0·014 | 0·026–0·073 | 0·049 | |
| Rheumatoid factor-positive | ELISA | 0·061 | 0·029 | 0·032–0·167 | 0·061 | |
| | ELFA | 0·041 | 0·020 | 0·025–0·088 | 0·050 | |
| Non-specific Hepatest agglutinations | ELISA | 0·097 | 0·139† | 0·037–0·556 | 0·061 | 0·92 |
| | ELFA | 0·059 | 0·119† | 0·030–0·479 | 0·056 | |
| HBs Ag carriers | ELISA | 0·158 | 0·068 | 0·080–0·308 | 0·172 | |
| | ELFA | 0·096 | 0·058 | 0·065–0·279 | 0·112 | |
| Patients with acute hepatitis B | ELISA | 0·77 | 0·312 | 0·060–1·219 | 0·656 | 0·95 |
| | ELFA | 0·767 | 0·320 | 0·092–1·10 | 0·641 | |

* Optical density measurements for ELISA and potentiometer readings for ELFA.
† These data had a very skewed distribution.

The figure shows the correlation between the results of the ELISA and ELFA with sera from patients with acute hepatitis B. The correlation coefficient was 0·95, $p < 0·001$. The results of the assays of sera from the HBsAg carriers together with those that gave non-specific agglutination in the Hepatest showed a similar close correlation, $r = 0·92$, $p < 0·001$. The correlation between assays on the control group was not so close, $r = 0·54$, $p < 0·01$.

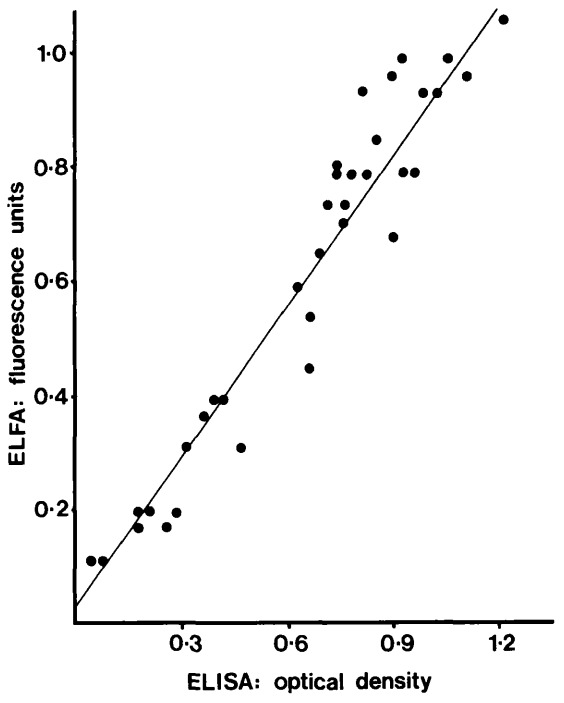


**Fig.** Correlation of the results of the enzyme-linked immunosorbent assay (ELISA) and the enzyme-linked fluorescent assay (ELFA) with sera from patients with acute hepatitis B. The least squares regression line is shown.

## Discussion

This study has shown that the results obtained by the new ELFA with peroxidase are closely comparable with those obtained by a standard ELISA technique. With sera from patients with high levels of IgM antibody to HBcAg the correlations were very close. The sensitivity of the two tests was similar under the conditions used, although a 10-fold dilution of the reaction mixture from the ELFA was necessary to obtain a sufficient volume for the fluorometer used. The use of alternative equipment, such as the Microfluor Automatic Reader (Dynatech) or the Titertek Floroskan (Flow Laboratories) with the appropriate filters would remove the need for such dilution.

We did not stop the reaction in the ELFA with acid because the reduction of the *p*H quenched the fluorescence. We did not search for an alternative enzyme inhibitor but, if the plates are read rapidly in automatic equipment, such inhibition is unnecessary.

The fluorescent product of *p*-hydroxyphenylacetic acid was, as claimed by Guilbault *et al.* (1968), very stable and was unaffected by light.

Doubtless the ELFA described here is open to considerable technical improvement but, even in its present form, it appears to be a useful addition to the range of sensitive and reliable antibody assays.

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