

A NEW FORMAT ELISA FOR THE DETECTION OF HBsAg

Rolando Ochoa Azze

DACTA, Instituto Finlay, Ave. 27 No. 19805, AP 16017, La Lisa CP 11600, La Habana, Cuba.
Fax: (53-7) 33 6754; E-mail: inmunoquimica@finlay.edu.cu

ABSTRACT

A new ELISA has been standardised, using two monoclonal antibodies directed against HBsAg. The sheep anti-HBsAg/alkaline phosphatase conjugate and the positive control were dehydrated at 37 °C for 18 h and 35% relative humidity. Sucrose was used as stabiliser with an optimum concentration of 125 mg/mL. The one step ELISA is carried out by adding a washing solution into control wells and samples into the remainder. Sensitivity and specificity were 100%. Discrimination value was 0.22 U/mL. Stability was studied for coating antibodies, dehydrated conjugate and the dehydrated positive control. The stability during their use was over 80% at 150 days and storage stability at 2-8 °C was over 80% a year after.

Keywords: conjugate stability, HBsAg, HBsAg stability, new format ELISA, one step ELISA

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RESUMEN

Se estandarizó un ELISA en el que se usan dos anticuerpos monoclonales de captura anti-HBsAg de diferente especificidad. El conjugado anti-HBsAg (ovino)/fosfatasa alcalina y el control positivo se presentan desecados en los pocillos mediante incubación por 18 h a 37 °C y humedad de 35%. La concentración óptima de sacarosa empleada como elemento estabilizador fue de 125 mg/mL. Para el ELISA en un paso de reacción se requiere tan sólo añadir solución de lavado en los pocillos correspondientes a los controles, las muestras se adicionan en los pocillos restantes. La sensibilidad y especificidad fueron del 100%. El valor de discriminación de 0,22 U/mL. La estabilidad de uso y en anaquel (2-8 °C), fue superior al 80% a los 150 días y al año respectivamente, tanto para los anticuerpos de recubrimiento como para el conjugado y el control positivo desecados.

Palabras claves: ELISA de nuevo formato, ELISA en un paso, estabilidad de conjugados, estabilidad del HBsAg

Introduction

In recent years, non-competitive solid-phase enzyme immunoassays (ELISA), based on a one-step sandwich principle, have been developed in order to simplify the technical procedures. The simultaneous addition of sample and conjugate is possible because of non-interference between immobilised molecules (antigens or antibodies) and conjugates (antibody/enzyme or antigen/enzyme) [1-6]. These assays achieve high sensitivity without a reduction in specificity [4, 5, 7].

Immunochemical assays have also been developed, with enzymes and depot substrates or coloured particles. These assays are read visually and require no laboratory equipment. Quality controls can also be included in an assay [8, 9].

We decided to develop a method that combines the advantages of new generation ELISA and some of the benefits of immunochemical assays. We have produced an ELISA for the detection of HBsAg with the conjugate and positive and negative controls dehydrated into the wells of the polystyrene plate.

Materials and Methods

Conjugate

A sheep anti-HBsAg/alkaline phosphatase (AP) conjugate made in the Immunoassay Centre (IAC), Havana, Cuba, was used at 0.28 µg/mL, and was diluted in a 0.05 M tris buffer solution, 0.15 M NaCl, 0.5 mg/mL NaN₃, 0.05% tween 20 (v/v) pH 8, containing 10 mg/mL of bovine albumin

(Merck, Germany) and 10% (v/v) of normal sheep serum IAC. Sucrose (Merck, Germany.) was added to a concentration of 500, 250, 125, 62.5 and 31.25 mg/mL. The selected concentration was that which achieved the best discrimination between HBsAg negative and HBsAg positive samples of 2 and 1 U/mL (from the Paul Ehrlich Institute, Germany (PEI)).

Controls

Liquid positive control (PC), was produced from heat inactivated human serum containing 44 µg/mL of the HBsAg subtype ad IAC. It was stabilised in a 0.02 M tris buffer solution, 0.15 M NaCl, 2 mg/mL NaN₃ pH 7.2, containing 80 mg/mL bovine albumin (Merck, Germany) and was prepared to a final concentration of 6 U/mL. The dehydrated positive control (DPC) was made with the same reagents. It was mixed with the conjugate to a final concentration of 6 U/mL.

The negative control (DNC) was the dehydrated conjugate added to the wells after the dilution with the buffer solution (0.015 M tris, 0.15 M NaCl, 0.2 mg/mL NaN₃, pH 7.8 (TBS) with 0.05% (v/v) tween 20). The liquid negative control was the HBsAg negative human serum (NHS) from the UMELEISA HBsAg[®] kit IAC.

Liquid and dehydrated controls were compared by analysing 20 replicates with the Student's T test, previous determination of the normality of each distribution was carried out using the Kolmogorov Smirnov test [10].

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Preparation of reaction plates

a) *Plates.* MaxiSorp® 8 x 12 wells polystyrene (PS) plates (Nunc, Denmark).

b) *Capture antibodies.* Two different monoclonal antibodies anti-HBsAg to epitope "a"; Hep 1 (IgG2b) (Center for Genetic Engineering and Biotechnology, Havana, Cuba) [11, 12] and Hep 8 (IgG1) IAC [13], were diluted to 10 µg/mL and 5 µg/mL, respectively, in a buffer solution (0.05 M tris, 0.2 mg/mL NaN₃, pH 8.0). 100 µL of each solution per well was added and plates were incubated at 25 °C for 18 h in a humidity chamber. Unbound antibodies were removed by washing (1 x 30 seconds) with 300 µL per well of 0.15 M phosphate buffered saline (PBS), pH 7.3 with tween 20 0.05% (v/v).

c) *Blocking and stabilisation.* 300 µL of bovine albumin (Merck, Germany) 1 mg/mL and sucrose (Merck, Germany) 50 mg/mL in PBS were added to each well. Plates were incubated at 25 °C for 2 h.

d) *Addition of controls and conjugate.* After aspiration of the blocking solution, 50 µL of the conjugate was added to each well of columns 2-12. 50 µL of the positive control plus conjugate (DPC) was added to each well of the first column. Plates were dried at 37 °C for 18 h at a relative humidity of 35%.

e) *Storage.* Plates were sealed with silica gel in nylon bags and they were stored between 2-8 °C.

Test procedures

100 µL of the washing solution (TBS with 0.05% tween 20) was added to each well of the first column and to wells assigned as DNC. 100 µL of the sample was added to the remaining wells. Each well was mixed by pipetting. Plates were incubated for 2 h at 37 °C. Unbound samples and controls were removed after six washes. Inverted plates were tapped a few times on a paper towel and 100 µL of p-nitrophenylphosphate (Sigma) 1 mg/mL was added to each well in 0.92 M diethanolamine buffer pH 9.8. Plates were incubated for 30 min at 37 °C, and then the absorbances at 405 nm were read with a PR-521 reader IAC.

Cut-off

This was calculated using 100 serum samples from HBsAg negative blood donors. The mean (N) absorbance of DNC was subtracted from the absorbance of each sample. 100 standardised values were derived and the largest (k) was chosen as the cut-off value [14].

Sample values (S) were considered positive when S-N > k.

Quality parameters

a) *Diagnostic sensitivity and specificity.* These were estimated using three panels of sera previously tested by UMELISA HBsAg® and HBsAg Confirmatory Test® IAC. The first panel consisted of 80 HBsAg negative and rheumatoid factor positive serum samples (from the Rheumatology Institute, Havana, Cuba), the second panel of 20 serum samples from HBsAg positive blood donors and the third one of 4 HBsAg positive serum samples (PEI) between 2 and 0.25 U/mL. The sensitivity and specificity was calculated according to the following formulae [15].

$$\text{Sensitivity \%} = \frac{\text{HBsAg positive samples correctly classified}}{\text{HBsAg positive samples in panels}} \times 100$$

$$\text{Specificity \%} = \frac{\text{HBsAg negative samples correctly classified}}{\text{HBsAg negative samples in panels}} \times 100$$

b) *Discrimination value and width of the "grey zone".* HBsAg positive samples of 2, 1, 0.5, 0.25, 0.125 and 0.0625 U/mL were produced by two fold dilutions of an HBsAg positive serum PEI with NHS. They were analysed daily for 20 days and the width of the "grey zone" (between 5 and 95% of the positive results) and the discrimination value (50% of positive results) were calculated [16].

c) *Stability during use.* The stability of the reaction plates (coating antibodies and dehydrated conjugate) was evaluated by repeated opening of the nylon bag, extraction of strips and re-closure. The stability was determined by comparing the mean absorbance of PC in these strips with the absorbance of PC in control strips. The stability of DPC was determined using the same methodology. The stability of the reaction plates and DPC was assessed at 30, 60, 90, 120 and 150 days. It was calculated as follows [13].

$$\text{DPC stability \%} = (\text{tested DPC/control DPC}) \times 100$$

$$\text{Plates stability \%} = (\text{tested PC/control PC}) \times 100$$

Stability above 80% was considered good.

d) *Storage stability.* The stability of the DPC and reaction plates stored at 2-8 °C was determined by comparing the mean absorbance detected in testing at 6, 9 and 12 months with the absorbance detected on day one. Storage stability was calculated as follows [13].

$$\text{DPC stability \%} = (\text{tested DPC/initial reading}) \times 100$$

$$\text{Plates stability \%} = (\text{tested PC/initial reading}) \times 100$$

Stability above 80% was considered good.

Results and Discussion

Conjugates have previously been placed in the wells in some ELISA in order to simplify the technical procedure, e.g. Organon Teknika (Vironostika HIV Uniform II) which uses lyophilised spheres of conjugate [6]. We decided to employ dehydration in order to facilitate the production process and to simplify the sealing of the plates. We also used dehydrated controls. The DPC was calculated for a final concentration of 6 U/mL, to produce an absorbance distinguishable from the DNC. The mean absorbance of the DPC was slightly lower than the liquid control, and the mean absorbance value of the dissolved conjugate in the washing solution (DNC) was slightly larger than NHS (Table 1).

The best concentration of sucrose was 125 mg/mL, this achieved the best discrimination between HBsAg

Table 1. Statistical analysis between HBsAg positive and negative controls.

n = 20	Negative (liquid)	Negative (dehydrated)	Positive (liquid)	Positive (dehydrated)
Mean	0.08	0.09*	0.91	0.89**
CV (%)	12.80	15.20	2.00	3.00

CV: Coefficient of variation.

* p: 0.018

** p: 0.011

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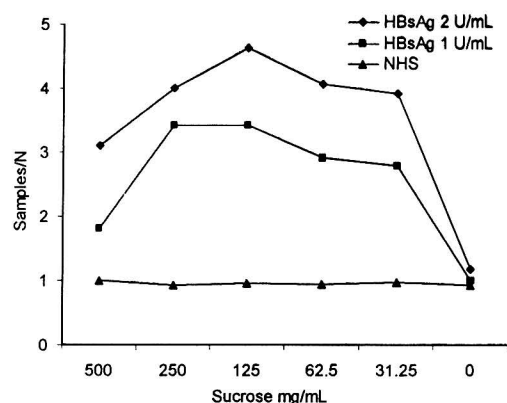


Figure 1. Determination of the optimum concentration of sucrose for the dehydrated conjugate. HBsAg negative (NHS) and HBsAg positive samples were standardised with the mean of negative controls (samples/N).

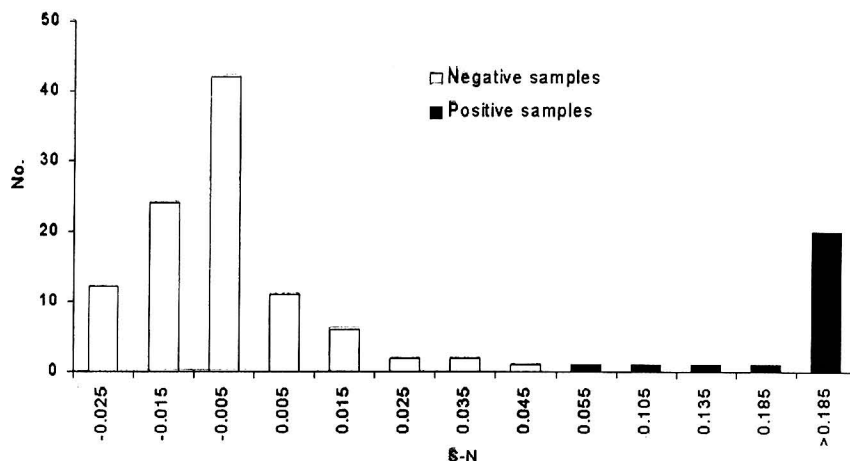


Figure 2. Distribution of frequencies in panels of HBsAg positive and negative samples, showed as the subtraction between samples and mean of negative controls (S-N). Cut-off S-N > 0.05.

negative and HBsAg positive samples of 2 and 1 U/mL (PEI) (Figure 1). However, it may be necessary to use a different sucrose concentration if different conjugates are used.

It is interesting that low concentrations of sucrose can block the adsorption of the conjugate to the PS surface. On the other hand, an excess concentration of sucrose could be harmful (Figure 1), because it may interfere with the antibody/antigen interaction. For that reason, the determination of the correct sucrose concentration is essential.

Large concentrations of sucrose, even 600 mg/mL, have been used in immunochromatographic assays as a support for the surface movement of the conjugate [8]. It should have had a similar role in our assay. Sucrose takes part in hydrogen bonding with chemical groups of the PS surface, especially on high binding surfaces, rich in hydrophilic groups, such as MaxiSorp® plates [17] and with hydrophilic macromolecules such as the conjugate. It hinders hydrophobic bonds and the stable binding of protein molecules. The binding of carbohydrates is not stable, but sucrose could be considered as a blocking agent. Also, its known stabilising role in the lyophilisation process, and the dehydration method (18-23) we use for the conjugate and the HBsAg positive controls, could allow its use in diverse immunoassays.

The dehydrated material remained firmly adherent to the bottom of the wells, which facilitated the manipulation of the reaction plates (strips), it also diluted quickly, which is important in the technical procedure.

The cut-off value was $S-N > 0.05$ ($S > N + 0.05$). We decided to use the method of limit value [14], calculated not with the absorbance values but standardised with the mean absorbance of DNC of each assay. We not only avoided false positive results, characteristic of this method [24], but also false negatives, which was demonstrated after correctly classifying HBsAg negative and positive panels (Figure 2). Therefore, the sensitivity and specificity was 100%.

The narrow "grey zone", between 0.1 and 0.5 U/mL, gives a high precision [16]. The discrimination value was 0.22 U/mL (Figure 3).

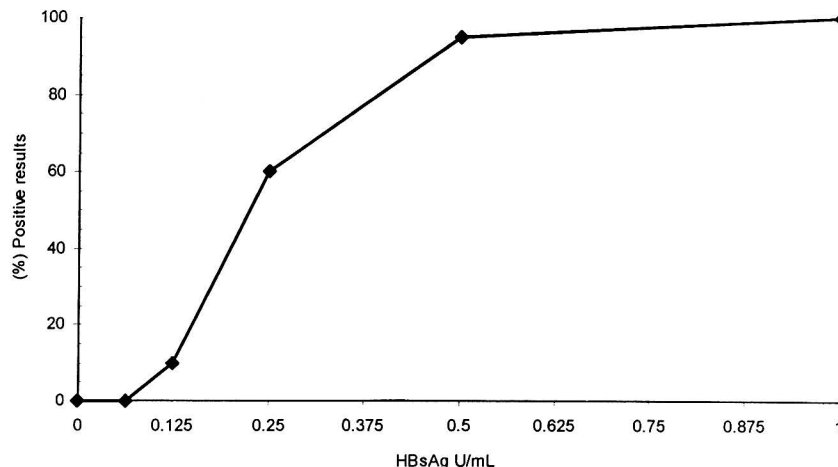


Figure 3. Estimation of the width of the grey zone (between 5 and 95% of the positive results) and discrimination value (50% of positive results).

Table 2. Stability during use by means of repeated opening of the nylon bag, extraction of strips and re-closing.

n = 24		Time (days)					
		0	30	60	90	120	150
Tested DPC	Mean	0.86	0.86	0.85	0.78	0.80	0.76
	CV (%)	4.10	3.10	4.00	2.80	4.60	4.70
Control DPC	Mean	0.93	0.91	0.92	0.88	0.91	0.92
	CV (%)	2.30	3.10	4.10	1.90	4.00	3.90
Tested PC	Mean	0.92	0.90	0.91	0.89	0.86	0.81
	CV (%)	3.10	2.60	6.00	4.90	2.10	8.40
Control PC	Mean	0.92	0.91	0.94	0.93	0.92	0.92
	CV (%)	3.10	6.00	6.30	6.00	3.10	6.10
DPC stability (%)			94.50	92.40	88.60	87.90	82.60
Plate stability (%)*			98.90	96.80	95.70	93.50	88.00

* It is referred to coating antibodies and the dehydrated conjugate.

Time 0 is the first reading.

DPC: dehydrated positive control.

PC: liquid positive control.

CV: coefficient of variation.

DPC stability (%): (tested DPC/control DPC) x 100.

Plate stability (%): (tested PC/control PC) x 100.

Table 3. Storage stability of the dehydrated positive control and reaction plates stored at 2-8 °C.

n = 16		Time (months)			
		0	6	9	12
DPC	Mean	0.86	0.83	0.81	0.76
	CV (%)	2.10	1.60	3.30	9.30
PC	Mean	0.89	0.89	0.86	0.82
	CV (%)	4.10	3.00	2.70	7.40
DPC stability (%)		96.50	94.20	88.40	
Plates stability (%)*		100.00	96.60	92.10	

* It is referred to coating antibodies and the dehydrated conjugate.
 Time 0 is the first reading.
 DPC: Dehydrated positive control.
 PC: Liquid positive control.
 CV: Coefficient of variation.
 DPC stability (%): (tested DPC/initial reading) x 100.
 Plate stability (%): (tested PC/ initial reading) x 100.

The use of the "strip by strip" method is important if small sample numbers are to be tested. We found excellent stability, higher than 80% at 150 days, for DPC and plates (Table 2). This stability is better than those previously reported [1-3, 6].

The storage stability (2-8 °C) of the plates and DPC was good at 12 months (Table 3).

We believe sucrose to be very important for good stability. The activity of the proteins decreases with the lyophilisation and the dehydration [18-23]. In order

to avoid this phenomenon many additives have been used, such as carbohydrates, which substitute water in order to stabilise proteins by means of hydrogen bonds [18-22]. Sucrose has demonstrated its role as a stabiliser, and this is reinforced in this investigation.

The stability should be similar for other dehydrated conjugates at least those from a sheep source. We have found similar behaviour in conjugates with horseradish peroxidase enzyme (unpublished data) and we believe that other markers should be studied. Controls should be carefully selected for each assay, positive control must be dehydration resistant like HBsAg.

This method not only simplifies the technical procedure but should also reduce the cost of a diagnostic kit because less equipment is required. An ELISA kit would only require 3 bottles; for the washing solution, the substrate and the substrate buffer. With coloured particles only one bottle would be sufficient. Therefore, this new method could be attractive for other immunoassays.

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