RESEARCH

Correlation of in vivo-in vitro *potency assays for the cuban Hepatitis B vaccine*

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ABSTRACT

Because of its advantages, there is a current trend to replace the *in vivo* methods by *in vitro* methods. The Center for Genetic Engineering and Biotechnology has developed an *in vitro* assay to determine the potency of the Recombinant Hepatitis B Vaccine with the aim of substituting the in vivo assay, thus eliminating its disadvantages regarding assay time, number of animals and variability of the results. Our objective was to study the use of the *in vitro* assay to assess the potency of the Cuban Hepatitis B Vaccine for quality control through its correlation with the *in vivo* potency. Formulated samples were evaluated by both methods at different concentrations of HBsAg and the Pearson correlation coefficient was calculated. A total of 213 batches were evaluated by both methods and the percentage of coincidence in complying with the specification for the *in vivo* and *in vitro* potencies was analyzed. The results of the study were a correlation coefficient equal to 0.87 and 100% coincidence in complying with the specification by both methods. Hence, we can conclude that the *in vivo* and *in vitro* potency assays are highly correlated and the latter can be used in quality control to release the vaccine.

Keywords: HBsAg, correlation, *in vivo*, *in vitro*, potency

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RESUMEN

Correlación de los ensayos de potencia *in vivo-in vitro* para la vacuna cubana contra la Hepatitis B. En la actualidad existe la tendencia a remplazar los mètodos *in vivo* por mètodos *in vitro*. En el CIGB se desarrolló un ensayo *in vitro* para determinar la potencia de la vacuna recombinante contra la Hepatitis B con el objetivo de sustituir el ensayo *in vivo* y de esta manera eliminar las desventajas de este último en cuanto a tiempo de ensayo, número de animales y variabilidad de los resultados. El objetivo de este estudio fue investigar la utilidad del método *in vitro* para la evaluación de la potencia de la vacuna cubana anti-Hep B como parte del control de calidad de la misma, mediante un estudio de correlación con el método de potencia *in vivo*. Para ello se evaluaron por ambos métodos muestras adyuvadas a diferentes concentraciones de HBsAg y se calculó el coeficiente de correlación de Pearson, también se evaluó un total de 213 lotes por ambos métodos y se analizó el porciento de coincidencia en el cumplimiento de la especificación para la potencia *in vitro* e *in vivo*. Los resultados del estudio fueron un coeficiente de correlación igual a 0.87 y un 100% de coincidencia en el cumplimiento de ambas especificaciones, por lo que se concluye que las técnica de potencia *in vivo* e *in vitro* correlacionan y esta última es adecuada para ser utilizada en el control de calidad y en la liberación de la vacuna

Palabras claves: HbsAg, correlación, *in vivo*, *in vitro*, potencia

*I*ntroduction

The production of a vaccine for human use must comply with quality control regulations [1] in which immunogenicity, generally evaluated by a potency assay in animals, is essential.

In biological assays, for potency determination in vaccines, there is a current trend for an alternative method that can substitute the use of animals, due to the wide diffusion of the 3R concept (Reduction, Refinement and Replacement) and its disadvantages in relation to cost and the variability of results introduced by animals. The replacement of assays in animals by the *in vitro* technique is both scientifically and economically advantageous.

In order to establish alternative methods WHO demands that the consistency of production must be demonstrated and the proposed alternative method validated and correlated to the standing method [2].

An alternative method for Potency determination with the Recombinant Hepatitis B Vaccine was developed in the Quality Control Laboratories of the Center for Genetic Engineering and Biotechnology. This method may be used for the quality control of the vaccine and is less costly, less variable and of short duration [3].

The alternative of using the *in vitro* potency assay instead of the *in vivo* assay was accepted by the European Pharmacopoeia in 1995 [4], as long as the correlation between both assays is presented to the national control authority. From the regulatory point of view the most relevant issue is that the alternative method should not approve a product that was rejected by the standing method.

The validation of an alternative quantitative method is complicated, due to the absence of products with high and low potency. Manufacturers assure a consistent quality of their product and these extremes are not obtained during production. That is why this study was aimed to investigate the usefulness of the

1. WHO. Requirements for hepatitis B vaccines made by recombinant DNA techniques. Technical Report Series, No. 889, 1999. Annex 4. Page 95.

2. Brown F, Hendriksen C, Sesardic D (eds). Alternatives to Animals in Development and Control of Biological Products for Human and Veterinary Use. Dev Biol Stand Basel, Karger 1999;101:13-7.

3. Rodríguez O, García G, Izquierdo M, Quintana M, Marcelo J. *In vitro* assay for the cuban vaccine for Hepatitis B. Applied Biotechnology 2001;18(3):154-8.

4. European Pharmacopoeia 1995; 1056:1-7.

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in vitro method to evaluate the potency of the Cuban Hepatitis B Vaccine as a part of its quality control through a correlation study with the *in vivo* potency assay. Formulated samples were evaluated by both methods at different concentrations of HBsAg and the Pearson correlation coefficient was calculated. A total of 213 batches were evaluated by both methods and the percentage of coincidence in complying with the specification for the *in vivo* and *in vitro* potencies was analyzed.

The results of the study show a correlation coefficient of 0.87 and a 100 % coincidence in complying with the specification by both methods. Hence, we can conclude that the *in vivo* and *in vitro* potency assays have a high correlation and the latter can be used in the quality control of the vaccine.

Materials and methods

Materials

The neutralizing antibody used in the *in vitro* potency assay is a purified human polyclonal antibody at a concentration of 1000 IU/L. This material was calibrated against the International Reference Material distributed by the Biological Standards Laboratory in Amsterdam.

The Reference Material of the Hepatitis B Vaccine comes from a production batch that was calibrated against the Reference Material mentioned above and the International Reference Material of the vaccine.

The experimental samples prepared at different concentrations of HBsAg (30, 20, 15, 12.5, 10, 5, 2.5, 1.25 mg) were supplied by the Formulation and Packaging Department of the Center for Genetic Engineering and Biotechnology.

The vaccine batches that were evaluated were manufactured by the Center for Genetic Engineering and Biotechnology in Havana, Cuba (HEBERBIOVAC HB).

Methods

In vivo potency assay

The *in vivo* potency assay (measures immunogenicity) was carried out according to WHO Technical Series, 1989 [5], and consists of inoculating 5 doses of the reference preparation and 5 doses of the sample in balb/c, haplotype $h^{2d,q}$ mice (10 mice per dose). Another 10 mice were inoculated with placebo. Twenty eight days after the inoculation, the mice were bled by the retroorbital route and blood samples from each animal were collected separately. The samples were centrifuged to extract the sera, which were evaluated to determine the presence of antibodies against Hepatitis B through ELISA [6]. The potency value was obtained using a program of parallel lines, which performs a Probit transformation of the percentages of seroconversion in each dose. [7].

ELISA for anti-Hepatitis B antibody determination

PolySorp (NUNC) plates of 96 wells were sensitized with the surface antigen of recombinant hepatitis B diluted with bicarbonate carbonate buffer and incubated at 37 ºC for two hours. Afterwards, the plates were washed three times with PBS + 0.05% Tween 20. The plates were then blocked with 2% bovine seroalbumen and incubated at 37 ºC for 1 hour. The samples, placebo and positive and negative controls were applied and incubated at 37 ºC for two hours. The conjugate of the test was the surface antigen of recombinant Hepatitis B coupled to pungent radish peroxidase. This substance was added to the plates and incubated for 1 hour at 37 ºC. o-phenylenediamine with 30% hydrogen peroxide was used as the test substrate that was incubated for 15 minutes at room temperature in the dark and the reaction was stopped with 2N sulfuric acid. The plates were read at 492 nm.

The plates having an optical density that was higher than or equal to the mean of the placebos multiplied by 1.5 were considered positive.

In vitro potency assay

The *in vitro* potency assay (measures antigenicity) was carried out according to the following procedure: Four dilutions of the reference material and samples were prepared (1/8; 1/16; 1/32; 1/64) with three separate replications, while having a maximum control (1 000 IU/L of the neutralizing antibody) and a minimum control (1 mL of the vaccine). The neutralizing antibody was added to the samples, to the reference material and to the maximum control and they were incubated at 4 ºC for 20 to 24 hours. The samples and the control were centrifuged at 10 000 rpm for 5 min to eliminate the antibody that had coupled with the antigen. The supernatant from centrifugation was evaluated through ELISA [6] to determine the amount of free antibodies. Potency was calculated by a parallel lines program using a Logit transformation to make the dose-response curves linear [7].

Correlation of the methods

The correlation between the *in vivo* and *in vitro* techniques was evaluated in two ways

The first was to evaluate batches manufactured for three years (213 batches) by both techniques and to analyze the percentage of coincidence in their compliance with the specification.

The second was to prepare experimental formulations containing different amounts of antigen (30, 20, 15, 12.5, 10, 5, 2.5, 1.25 mg) and to evaluate them by both methods. The results obtained for each sample by each method were averaged according to USP 23 page 1713-1714 [8]. The averages obtained were logarithmically transformed for the correlation analysis. The correlation coefficient was calculated by the Pearson formula [9, 10], and its confidence limits by the Fisher method [9, 10].

Calculating tools

The *in vivo* potency was calculated by a program developed in our laboratory, which uses the method proposed by Finney for the assays of the "quantal" response [7]. This program performs a "Probit" transformation of the percentages of seroconvertion while it logarithmically transforms the inoculated doses. The transformed data from the sample and the reference material were adjusted to two parallel lines. Also, besides estimating the potency, this program performs significance tests for linearity and parallelism and estimates the confidence interval of the calculated potency.

5. WHO/BS/86. Proposed requierements for the Hepatitis Bvaccine made by recombinant DNA techniques, 1986;1487(2).

6. Ramirez V, Gonzàlez-Griego A, Alerm A, Izquierdo M, Peña M, Vega I, *et al*. Immunoenzymatic assay for the quantification of an anti-HBs antibody. Applied Biotechnology 1993;10:102.

7. David J Finney. Statistical methods in biological assay. 3rd ed. London: Charles Griffin and Co Ltd;1978.

8. USP 23, 1995;1713-4.

9. Robert GD Steel, James H Torrie. Biostatistics: Principles and procedures. 2nd ed. 1988.

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The Parlin program was used to process the primary data from the in vitro potency assay. This program was also developed in our laboratory. It uses the algorithm proposed by Finney for assays with a quantitative response [7]. A "Logit" data transformation is used to calculate the *in vitro* potency. Parlin performs the significance tests for linearity and parallelism and estimates the confidence interval for potency.

Results and discussion

The number of batches analyzed, the percentages of compliance with the specification in both assays and the percentage of coincidence between the assays are shown in table 1. It is observed that from a total of 213 batches, there was a 100% coincidence in complying with the specification by both techniques. This measures the consistency of production from the viewpoint of immunogenicity as well as antigenicity, where the molecule and the formulation process play an important role.

The correlation coefficient obtained from the analysis of the samples formulated at different concentrations of HbsAg was 0.87 with limits from 0.44 to 0.98. This means that the regression is highly significant because the lower limit is far from zero, while its upper limit is very near the maximum value, one. In order to graphically illustrate the results, a chart of the correlated variables was drawn after being standardized with their corresponding standard deviation. This chart is shown in figure 1.

The results of the experimental formulations are shown in table 2. It is observed that when the sample does not comply with the specification by the *in vivo* method, it does not comply by the *in vitro* method either. This is a very important requirement for alternative methods, since from the regulatory viewpoint the most important issue is that the alternative method cannot approve a product that was rejected by the standing method.

Conclusions

The fact that a 100% coincidence was obtained for 213 batches shows that the production is consistent and there are no batches below the specification limit, this being a characteristic of the batches produced under normal conditions.

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Table 1. Percentage of coincidence in complying with the specification of the *in vivo* and *in vitro* assays

Number of batches	Complying with the specification	Complying with the *in vivo* specification	Complying with both specifications
213	213	213	213
Percentage	100	100	100

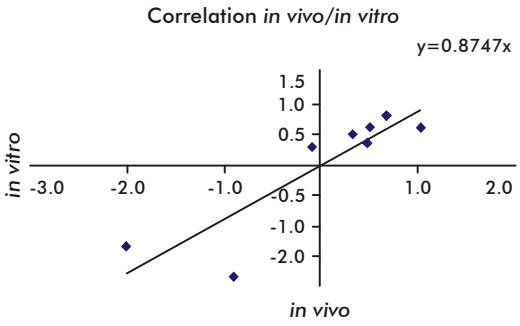

Figure 1. Chart of the correlation of the *in vivo* and *in vitro* potency assays.

Taking into account the above results, we can conclude that the *in vivo* and *in vitro* potency assays show a high correlation. Therefore, the latter can be used with a high reliability for the quality control of the cuban vaccine against Hepatitis B.

Table 2. Results of the experimental formulations by the *in vivo* and *in vitro* potency assays

Concentration (μg)	Relative potency *in vitro* (times)	Relative potency *in vivo* (times)
30	1.627	0.9
20	1.087	1.4
15	0.88	0.9
12.5	0.85	0.5
10	0.718	0.7
5	0.441	0.424
2.5	0.173	0.003
1.25	0.047	0.01