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RESEARCH

Preformulation study of a Cuban pentavalent DPT-HB-Hib vaccine, Heberpenta®-L

✍Néstor S Expósito-Raya[1], Yayrí C Prieto-Correa[1], Mabel Izquierdo-López[2], Zoe Nuñez-Lamothe[2], Soraya Piloto-Díaz[2], Yamila Martínez-Cuellar[2], Makis Torres-Toledo[2], Annette Pereira-Sanabria[2], Rubén López-Edghill[2], Jose L Marcelo-Sainz[2], Amarilys Rodriguez-Arrebato[2], María de los A Denis-Cruz[2], Yanet Alderete-Avalos[2], Anaelsi Chacon-Molina[2], Eduardo Martínez-Díaz[3]

[1] Dirección de Desarrollo Tecnológico, Centro de Ingeniería Genética y Biotecnología, CIGB Ave. 31 e/ 158 y 190, Cubanacán, Playa, CP 11600, La Habana, Cuba
[2] Dirección de Control de Calidad, CIGB, La Habana, Cuba
[3] OSDE Biocubafarma, Avenida Independencia No. 8126 esq, a 100. Boyeros, La Habana, Cuba
✍nestor.exposito@cigb.edu.cu

ABSTRACT

The development of combined vaccines is a very useful project for all the advantages that this type of vaccine provides. However, complexities in its execution due to their technological challenges make difficult its development. In this sense, the pentavalent vaccine (DPT-HB-Hib), branded as Heberpenta®, has been produced in Cuba containing five antigens: diphtherial and tetanus anatoxins, whole cells of *Bordetella pertussis*, recombinant hepatitis B virus surface antigen and the synthetic polysaccharide (polyribosyl ribitol phosphate, PRP) of *Haemophilus influenzae* conjugated with tetanus anatoxin (PRP-T). A pre-formulation study was carried out using a mixture of adjuvants defining the optimum absorption time for each antigen. Three lots formulated at a pilot scale with a defined technology were studied using different physical-chemical and biological tests. The technology for the formulation of the pentavalent vaccine produced in Cuba was defined according to these results, which must later undergo stability studies and preclinical and clinical studies.

Keywords: Pentavalent vaccine, adsorption kinetics, vaccine potency, vaccine immunogenicity, preformulation

RESUMEN

Estudio de preformulación de una vacuna pentavalente de DPT-HB-Hib cubana, Heberpenta®-L. El desarrollo de vacunas combinadas es un proyecto muy útil por todas las ventajas que ofrece este tipo de vacuna, sin embargo, no está exento de complejidades en su ejecución debido a sus desafíos tecnológicos. En Cuba se desarrolló satisfactoriamente una vacuna pentavalente (DPT-HB-Hib), bajo la marca Heberpenta®, que contiene cinco antígenos: anatoxina diftérica y tetánica, células enteras de *Bordetella pertussis*, antígeno de superficie recombinante del virus de la hepatitis B y el polisacárido sintético (polirribosil ribitol fosfato, PRP) de *Haemophilus influenzae* conjugado con anatoxina tetánica (PRP-T). Se realizó un estudio previo a la formulación utilizando una mezcla de adyuvantes que definen el tiempo de absorción óptimo para cada antígeno. Se formularon tres lotes a escala piloto con la tecnología definida y se estudiaron utilizando diferentes pruebas físico-químicas y biológicas. La tecnología para la formulación de la vacuna pentavalente producida en Cuba se definió de acuerdo con estos resultados, que luego deben someterse a estabilidad, estudios no clínicos y clínicos..

Palabras clave: Vacuna pentavalente, cinética de adsorción, potencia de la vacuna, inmunogenicidad de la vacuna, preformulación

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Introduction

The development of combined vaccines has been very useful to reduce the number of vaccination schedules, due to the subsequent increase in the number of independent vaccines recommended for children during the first two years of life, and thereby the number of punctures, visits to medical offices, and have made hard to achieve high infant population coverage [1].

The benefits of combined vaccines consist of reducing vaccination schedules, reducing trauma in children, achieving better vaccination compliance [2-4]. It also reduces administrative costs, less storage space is needed, therefore, new vaccines can be included in the vaccination schedules [5]. In Cuba, the development of combined vaccines has been promoted from existing vaccines such as triple DPT, monovalent recombinant hepatitis B (HB) (Heberbiovac HB®) and the first synthetic vaccine in the world against *Haemophilus influenzae* type b conjugated to tetanic toxoid (PRP-T), Quimi Hib®.

The availability of these vaccines paved the way for the combination of the DPT vaccine with HB, managing to register and apply, in 2004, the tetravalent vaccine (DPT-HB) Trivac HB, not only in Cuba, but also in other countries. Later, in 2010, the pentavalent DPT-HB-Hib, Heberpenta®-L vaccine was obtained and included in the Cuban PAHO Child Immunization Program . In this article, we will approach an overview of the development and proposal of a production technology for the Cuban DPT-HB-Hib pentavalent vaccine.

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*M*aterials and methods

Antigen sources

In the experimental batches where the adsorption of the D, P, T and HB antigens were studied, the aluminum hydroxide adjuvant, Alhydrogel 3% (Danish company Brenntag Biosector Ltd.) was used, due to its patent-free technological alternative that it is not protected in the combination vaccine patent [6]. Polyribosyl ribitol phosphate conjugated to tetanus anatoxin was adsorbed on aluminum phosphate, AdjuPhos 2 % (Danish company Brenntag Biosector Ltd.). Tetanus anatoxin at a concentration of 20 limit of flocculation (Lf)/mL, diphtherial anatoxin at 50 Lf/mL, HBsAg at 20 µg/mL, PRP-T at 20 µg/mL and whole cell *Bordetella pertussis* at 32 opacity units (OU)/mL.

To calculate the percentage of adsorption of each antigen in the formulations, the equation used was:

Adsorption % = (A – B × 100) / A

Where:

A: Amount of antigen added.

B: Amount of antigen identified in the vaccine supernatant by the method used.

Active pharmaceutical ingredients

The active pharmaceutical ingredients (API) tetanus anatoxin (T), diphtherial anatoxin (D), whole cells of *B. pertussis* (P) produced at the Finlay Institute for Serums and Vaccines were used in each formulation. The recombinant hepatitis B surface antigen (HBsAg) and the polyribosyl ribitol phosphate conjugated to tetanus anatoxin (PRP-T) were produced by the Center for Genetic Engineering and Biotechnology, CIGB (Habana, Cuba).

Selection of adjuvants

The most important criterion for the selection of an adjuvant for human vaccines was biosafety; hence, aluminum compounds were selected as the only adjuvants licensed for human use [7]. Phosphate and aluminum hydroxide adjuvants have been used in vaccines since 1926, widely demonstrating their safety; however, the mechanism for its immunostimulatory activity has been little studied. Its activity is currently considered to be related to the degree of adsorption of the antigen [8].

The degree of adsorption that would take place when the vaccines are formulated can be predicted knowing the isoelectric point (*pI*) of the antigen and the adjuvant; however, the use of these values may not be as effective in predicting the physical properties of a vaccine, since many factors can influence the physicochemical process of adsorption of the antigen in the production of adsorbed vaccines.

Adsorption dynamics of diphtherial anatoxin

Six formulation batches of 50-mL each were prepared. The aluminum hydroxide gel at 3 % was diluted to 1 % with 0.85 % saline, at pH 6.6, and sterilized at 121 °C for 15 min. The sterile purified diphtherial anatoxin was added at a concentration of 2500 Lf/mL in each formulation to reach a final concentration of 50 Lf/mL. The addition of diphtherial anatoxin was carried out with continuous dripping using a Gilson P-5 000 pipette. It was stirred gently and samples were taken at 30 min, 1, 2, 3, 4 and 5 h of stirring, respectively. The test was performed in duplicate at each sampling time.

Adsorption dynamics of tetanus anatoxin

Six formulation batches of 50 mL each were prepared. The aluminum hydroxide gel at 3 % was diluted to 1 % with 0.85 % saline, at pH 6.6, and sterilized at 121°C for 15 min. Sterile purified tetanus anatoxin was added at a concentration of 1000 Lf/mL in each formulation to reach a final concentration of 20 Lf/mL. The addition of tetanus toxoid was carried out with continuous dripping using a Gilson P-5 000 pipette. It was stirred gently and samples were taken at 30 min, 1, 2, 3, 4 and 5 h of stirring. The test was performed in duplicate at each sampling time.

Assay to determine the concentration of diphtherial and tetanic anatoxins in the vaccine supernatant

The presence of diphtherial and tetanus anatoxins in the vaccine supernatant was determined by the flocculation assay [9], mixing variable amounts of the anatoxin with constant amounts of the antitoxin under permanent observation and constant temperature. After the time established for each sampling 15 mL of the vaccine were taken. Samples were centrifuged (HITACHI SCT-15B, Tokyo, Japan) at 2000 rpm for 15 min, 10 mL of the supernatants were taken carefully to avoid dragging in the precipitated gel and the anatoxins were quantified. The mixture that flocculates first indicates the approximate amount of the anatoxin found in the sample, and it is expressed as the limit flocculation (Lf). The test quantified the amount of anatoxin that produces a white precipitate in the shortest possible time, when mixed with 1 IU of antitoxin [9, 10].

Adsorption dynamics of HBsAg

Five batches 50-mL each of the formulation were prepared. The aluminum hydroxide gel at 3 % was diluted to 1 % with 0.85 % saline, at pH 6.6, and sterilized at 121 °C for 15 min. HBsAg was added at a concentration of 1 mg/mL in each batch to reach a final concentration of 20 µg/mL. The addition of HBsAg was carried out with continuous dripping using a Gilson P-5000 pipette. It was stirred gently and samples were taken at 15 and 30 min, and at 1, 1.5 and 2 h.

Determination assay of HBsAg in the supernatant

One milliliter of the vaccine was taken, which was centrifuged at 2,000 rpm (HITACHI SCT-15B, Tokyo, Japan) for 15 min and the content of HBsAg in the supernatant was evaluated using the ELISA assay for the quantification of HBsAg [11]. The plate was sensitized with a polyclonal anti-HBsAg antibody obtained from ram plasma (solid phase); the sample was applied and then a horseradish peroxidase conjugated polyclonal antibody was added. Orthophenylenediamine was used as the chromogenic substrate for the reaction. Absorbance values at 492 nm were determined on an ELISA reader (Titertek Multiskan PLUS, Labsystem, Finland).

Adsorption dynamics PRP-T

Two PRP-T adsorption studies were performed. Five batches of formulation of 50-mL each were prepared.

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The Adju-Phos aluminum phosphate gel at 2 % was diluted to 1 % with 0.85 % saline. They were sterilized at 121 °C for 15 min. Once gels reached room temperature, their pH was measured and adjusted to 6.85. PRP-T was subsequently added at a concentration of 1 mg/mL in each batch to reach a final concentration of 20 µg/mL. The addition of the PRP-T was carried out with continuous dripping using a Gilson P-5000 pipette. It was stirred gently and samples were taken at 1, 2, 3, 4 and 5 h of stirring.

In the other test carried out, 6 batches of the formulation of 50 mL each, were prepared. The Adju-Phos aluminum phosphate gel at 2 % was diluted to 1 % with 0.85 % saline, and the pH was adjusted to 3.0. They were sterilized at 121 °C for 15 min. Once the gels reached room temperature, PRP-T was added at a concentration of 1 mg/mL in each batch to reach a final concentration of 20 µg/mL. The addition of the PRP-T was carried out with continuous dripping using a Gilson P-5 000 pipette. It was stirred gently, and samples were taken at 1, 2, 3, 4, 5, 6 h of stirring. At the time corresponding to 6 h before taking the sample, the pH was adjusted to 6.85 with a 0.2 M NaOH solution.

Determination test of PRP-T in the supernatant

Samples of 1 mL were centrifuged (Hitachi SCT-15B, Tokyo, Japan) at 10 000 rpm for 5 min and 600 µL of the supernatant were then taken.

In the case of the measurements of PRP-T, the pentose specific Orcinol method was used to quantify this antigen, by dehydration of the D-ribose in a strongly acid medium and the later formation of the derivative, which was stained through the oxidation of this product with Orcinol in the presence of iron salts. Finally, the samples were read to determine the absorbance at a wavelength of 670 nm in the Genesys 10UV spectrophotometer (Thermo Electron Corporation; Germany).

Adsorption dynamics of whole cells of *B. pertussis*

Seven formulation batches of 50-mL each were prepared. The aluminum hydroxide gel at 3 % was diluted to 1 % with 0.85 % saline, at pH 6.6. Whole and dead cells of sterile B. pertussis were added at a concentration of 250 OU/mL in each formulation to reach a final concentration of 32 OU/mL. The addition of *B. pertussis* was carried out with continuous dripping using a Gilson P-5 000 pipette. It was stirred gently and samples were taken at 3, 6, 9, 12, 18, 24 and 30 h of stirring.

Assay to determine the whole cells of *B. pertussis* in the supernatant

One milliliter was taken from each batch of vaccine corresponding to each sampling time. It was left to rest for 2 h, 1 µL of the supernatant was taken with great care to avoid dragging the precipitated gel. A 1/10 000 dilution was made, for this the 1 µL sample of the supernatant was diluted with 9.999 µL of water for injection for a volume total of 10 µL. From this diluted sample, 0.1 µL was taken and applied in the Neubauer chamber. To perform the cell count, a bright field microscope (Olympus, BH2, Japan) was used. The concentration of the cell suspension was determined by the formula.

Suspension concentration (cells/mL) = 10 000 (X/4) × dilution factor.

Where X stands for the number of cells counted.

Order of addition of the antigens in the formulation

The antigens were added in an ascending order according to their molecular weight to decrease a possible steric hindrance during the adjuvant adsorption process.

In the formulation process, the D and T antigens with molecular weights 62 and 150 kDa, respectively, respectively, were first adsorbed. The HBsAg in aluminum hydroxide and the PRP-T in aluminum phosphate were independently adsorbed, both antigens were mixed with D and T previously adsorbed, in order to protect the antigens from possible interactions. Finally, whole *B. pertussis* cells with a size between 0.5-2.0 µm were added.

Formulation of three 5-L batches of the pentavalent vaccine

According to the results obtained in the adsorption studies of each antigen, a formulation technology was used for the pentavalent vaccine that was evaluated through physicochemical and biological tests. Three batches of the pentavalent vaccine were made on a 5-L scale, designated as EPL0301E, EPL0302E, and EPL0401E.

Each batch of the pentavalent vaccine was formulated with different batches of API for the five active ingredients (Table 1), applying the principle of consistency of consecutive batches from the formulas:

D1 + T1 + P1 + HB1 + Hib1 = DPT-HB-Hib1

D2 + T2 + P2 + HB2 + Hib2 = DPT-HB-Hib2

D3 + T3 + P3 + HB3 + Hib3 = DPT-HB-Hib3

Once the three batches were obtained, a sampling was carried out to evaluate various parameters using the following tests.

Assay to determine the whole cells of *B. pertussis* in the supernatant

One milliliter was taken from each batch of vaccine corresponding to each sampling time. It was left to rest for 2 h.

Physicochemical tests

Determination of organoleptic characteristics

Each lot was sampled to evaluate organoleptic characteristics; a batch passed the test if the vaccine was

Table 1. Batches of active pharmaceutical ingredients (API) used in the formulation of the Cuban pentavalent vaccine

Vaccine batch	Diphtheria anatoxin	Tetanus anatoxin	*Bordetella pertussis*	HBsAg	PRP-T
EPL0301E	P2008DIF	P010/01	M3001PER	02MPAC326	53MPA0308T
EPL0302E	P2003DIF	P007/02	M3002PER	02MPAC327	53MPA0309T
EPL0401E	P3002DIF	P006/03	M3004PER	02MPAC336	53MPA0403T

HBsAg: Hepatitis B virus surface antigen. PRP-T: *Haemophilus influenzae* type b conjugated to tetanic toxoid

observed as a grayish liquid that was free from particles, which separated into two phases when at rest, with a gray white sediment (adjuvant) and a transparent supernatant that were readily resuspended when shaking [12].

pH determination

This method was based on the potentiometric determination of the hydrogen ion concentration in the product, measured through the use of electrodes and a Mettler Toledo Seven Easy pHmeter. The range of the proposed specification for this test was 6.4 to 7.4 [13].

Sterility

The sterility test was made according to the requirements of the USP 30 and it was based on microbial growth in the thioglycolate and tryptone-soy agar culture media. If no contamination was observed after 14 days, the sample passed the test in a satisfactory manner [13].

Aluminum ion content

The test used was based on the indirect determination of the aluminum hydroxide and phosphate through the quantification of the Al^{3+} located in these gels. The Al^{3+} forms a complex with the EDTA in an acetate regulating solution, valuating the excess of EDTA with pentahydrated cop-per sulfate II in the presence of the indicating solution of 1-pyridyl-2 azonaphthol. The vaccine passed the test if the Al^{3+} ion content was equal or less than 1.25 mg per single human dose (SHD) [14].

Thimerosal content

Thimerosal was indirectly determined in the vaccine tested by mercury spectrophotometric quantification, which forms a complex with the dithizone reagent. Separation was carried out through its extraction with chloroform. The specification proposed for this parameter is 0.005-0.02 g% of thimerosal per milliliter of the vaccine [15].

General safety

This test was carried out according to requirements of the British Pharmacopoeia, and its aim was to determine toxic reactions and weight loss in mice and guinea pigs after the intraperitoneal injection of 0.5 mL of the test sample per animal. The batch passes the test when no animal dies or shows no symptoms of the disease [12].

Specific toxicity of anatoxins and *B. pertussis*

Diphtheria anatoxin

The test is based on the observation of the signs shown by the animals inoculated with the tested samples, when free toxin is present because of a deficient detoxification or an anatoxin reversion process.

There were five guinea pigs of the same sex weighing 250-350 g in the test. Each one was inoculated by the intramuscular route with 5 mL of the vaccine, divided into two applications of 2.5 mL each. The animals were observed daily in the search for signs of intoxication with diphtherial toxin. The control group used purified diphtherial anatoxin.

The vaccine lot passes the test if no animal shows the characteristic signs of diphtheria for 6 weeks after the inoculation date, and if at least 80 % of the animals survive the test period [16].

Tetanus anatoxin

The same procedure was described above was followed, where the control group used purified tetanus anatoxin. The batches passed the test if no animal showed symptoms of specific paralysis or any other sign of tetanus within 4 weeks after the inoculation and if at least 80 % of the animals survived the test period [16].

Specific toxicity of B. pertussis

To evaluate the batches, 10 OF-1 mice of the same sex weighing 14-16 g were used for each sample, and a physiological saline solution was used for the control. The mice were injected by the intraperitoneal route with 0.5 mL of the vaccine being tested and were later observed [17].

The batch passed the test if:

- After 72 h the total weight of the group was not less than the weight before the injection.
- At the end of the seventh day, the average weight gain per mouse was not less than 60 % of that of the control group of mice.
- At the end of the test the death of the injected mice was not more than 5 %.

Identity tests

Identity of the diphtherial and tetanus anatoxins

For this test we used Ramon's identification method [9], by mixing variable amounts of the anatoxin with constant amounts of the antitoxin under permanent observation and constant temperature. The mixture flocculating the latter, indicates the approximate amount of the anatoxin found in the sample, which is expressed as the limit of flocculation (Lf). The test quantified the amount of anatoxin that produces a white precipitate in the shortest time possible, when mixed with 1 IU of the antitoxin [9, 10].

Identity of B. pertussis

The presence of the cells of *B. pertussis* is evaluated in formulations using specific sera against the three agglutinogens, pertussis toxin, filamentous hemagglutinin and pertactin, obtained in the laboratory after immunizing the rabbits with the purified antigens.

The pentavalent vaccine samples were mixed with sodium citrate at a concentration of 100 g/L, and later stored at 37 °C for 16 h; after that time they were centrifuged at 2000 rpm for 15 min to obtain a transparent supernatant liquid. From this supernatant, 50 µL were taken and mixed them in U-bottom plates (Nunc Maxisort) at serial dilutions with a factor of 2 of the specific sera previously diluted 1:4.

The plates were incubated for 24 h at 37 °C in a humid chamber. A positive agglutination well was that in which an agglutination clumping was formed. A negative control was incorporated that did not include the serum [18].

Identity of the HBsAg antigen

The identity test of the HBsAg was made in two stages: the separation of the HBsAg of the adjuvant gel and the identification of the antigen by ELISA.

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Five milliliters of the tested vaccine were taken and 400 mM of phosphate buffer saline (PBS) were added and shaken in a vortex (IKA MS1, Germany) for 1 min to deabsorpt the HBsAg from the adjuvant. Afterwards, 1 mL of the sample was centrifuged (Sigma 204, Germany) at 3500 rpm for 5 min; then, 200 µL of the supernatant was collected and the ELISA was carried out by coating the plates with 100 µL of a goat polyclonal antibody against HBsAg (006). The plates were incubated for 15 min at 50 ºC, and further washed three times with PBS plus 0.05 % Tween 20. After washing, 200 µL were taken from the standard solution and from the vaccine, and diluted 2 times with 7 mM PBS.

Subsequently, the samples were applied on the plate, incubated for 4 hours at 37 ºC in a humid chamber, washed as previously mentioned, and 100 µL of the anti-sheep peroxidase conjugate (1/100) were added to the plate. They were incubated for 1 h at 50 ºC, washed with 100 µL of the buffer solution of citrate-phosphate, containing the substrate of 1 mg/mL orthophenylendiamine and 30 % H_2O_2. The reaction was stopped by adding 50 µL of the stopping solution (2 M H_2SO_4). The plate was read at 492 nm in a Multiskan Plus Titertek. The result was positive if the HBsAg was detected in the vaccine tested [19].

Identity of the PRP-T

The qualitative method of immuno-identification by latex was used through a set of commercial reagents from Pastorex® Meningitis (61716). For the determination, a drop of the well homogenized vaccine was taken with a Pasteur pipette and mixed with a drop of latex sensitized with rabbit monoclonal antibodies that were specifically against the capsular polysaccharide of the *H. influenzae* type b bacterium. The test was considered positive if the sample agglutinated the positive control in the same way [20].

Biological tests

Potency of the diphtherial and tetanus anatoxins according to the FDA's indirect method

This procedure is based on the neutralization capacity of diphtherial and tetanus anatoxins found in the sera mixture of animals that had been immunized with the pentavalent vaccine, compared to the diphtheria and tetanus reference toxins.

Through the subcutaneous route, six Swiss albino guinea pigs of 450-500 g weight were immunized with 0.25 mL of the pentavalent vaccine (half of the total human immunizing dose). The animals were bled four to six weeks later and the test tubes were incubated at 37 °C for 2 h, after which the clots were separated from the walls of the test tubes and refrigerated at 5 ± 3 °C for clot retraction.

An equal amount of serum was taken from each animal and placed in one test tube (sera pool). For diphtherial anatoxin titration, the diphtherial anatoxin reference was diluted up to 1.0 IU/mL with a physiologic saline solution.

From the mixture of animal sera, 0.75 mL and 1.5 mL were taken and 5.25 mL and 4.5 mL of the gelatin buffer solution were diluted to 2 and 4 arbitrary units per milliliter (AU/mL), respectively.

A total of 0.5 mL of the diphtherial toxin was taken and diluted with 4.5 mL of the gelatin buffer solution, the operation was repeated in a test tube containing 9 mL of the gelatin buffer solution.

The mixtures were prepared with 3 mL of the diphtherial toxin and with the dilution of the serum-gelatin buffer solution; they were then maintained in the dark at room temperature for 1 h.

Two albino guinea pigs of 250-300 g were inoculated with 3 mL of each preparation through the subcutaneous route. They were observed daily for a week to evaluate symptoms and the death of animals due to diphtheria, and the IU of diphtherial antitoxin per milliliter of serum were detected.

The lot passed the test when reaching 2 or more IU of diphtherial antitoxin per milliliter of serum [21].

Tetanus anatoxin titration was carried out as follows. The standard tetanus antitoxin was diluted to 0.1 IU/mL with a physiologic saline solution. From the immunized guinea pig sera pool, 1 mL was taken and diluted with 9 mL of the physiologic saline solution. Then, 1.5 mL and 0.75 mL of the dilution of sera of guinea pigs were taken and diluted with 4.5 and 5.25 mL of the gelatin-buffer solution to 4 and 2 AU/mL, respectively.

Subsequently, 0.5 mL of the tetanus toxin were taken and diluted with 4.5 mL of the gelatin-buffer solution; this operation was repeated in a test tube containing 9 mL of the gelatin-buffer solution.

The mixtures were prepared with 3 mL of tetanus toxin and with the serum-gelatin buffer solution; they were kept in the dark, at room temperature for 1 h. Two albino guinea pigs of 350-400 g were inoculated with 3 mL of each preparation by the subcutaneous route.

They were observed daily for a week. The symptoms and death of the animals due to tetanus were observed and the IU of the tetanus antitoxin per milliliter of the serum was determined.

The batch passed the test if it reached 2 IU or more of the tetanus antitoxin per milliliter of the serum [21].

Potency of B. pertussis using the World Health Organization method

The pertussis potency of the vaccine was determined by the comparison of a working reference vaccine approved by the Quality Control Division of the Finlay Institute (Habana, Cuba; Reference lot VPR(1)/99), which was calibrated against the international standard for the pertussis vaccine.

Four dilutions of the reference vaccine and of each vaccine batch to be tested were made. The serial dilutions were prepared with a dilution factor that was no larger than five, for which a sterile 0.85 % sodium chloride saline solution was used.

Albino OF-1 mice of 10-18 g of weight were injected intraperitoneally with 0.5 mL of the dilution corresponding to each mouse in each immunization group. Afterwards, mice immunized with the reference and the test vaccines were injected with the challenge dose by the intra-cerebral route at a 14-17-days interval of after the immunization. The strain used for the challenge was *B. pertussis* 18 323.

To obtain estimates of the lethal dose (LD_{50}), dilutions of the challenge dose made (1:50, 1:250, 1:1 250) were inoculated within the brain of groups of

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control mice. The appropriate dilutions of the challenge dose were grown in a Bordet-Gengou agar base to determine the number of colony forming units (c.f.u.). The mice were observed for 14 days, and those dying within the first 72 h after the inoculation were excluded from the test.

The mice dying 72 h after the inoculation were recorded to determine the effective dose (ED_{50}) of the vaccines. ED_{50} for each preparation was determined by the Probit statistical method that assesses the linearity of the dose-response and the parallelism of the behavior of the vaccine in the test with the reference vaccine [22]. The value of the ED_{50} of each vaccine was set as the intermediate value between the highest and lowest immunizing dose and the regressions that did not show significant linearity and parallelism ($p \leq 0.05$). The challenge dose contained 100 to 1000 LD50 and no more than 300 c.f.u. The ED_{50} of the vaccine in the test and the standard vaccine were calculated by a method that offers an estimate of the limits of the 95 % confidence interval. The potency was estimated in terms of IU in the volume recommended for simple human dose (SHD).

The test vaccine complied with requirements for potency if the result of the test was statistically valid, showing that the estimated potency of the vaccine was not less than 4.0 IU per SHD [23].

Determination of the *in vivo* relative potency of the HBsAg

The potency test of the HBsAg was carried out according to the technical requirements of WHO [24]. For this, 1 mL of the test vaccine containing 20 µg/mL of HBsAg was diluted 1:16, 1:64, 1:256, 1:512 and 1:1 024 with aluminum phosphate gel at a concentration of 0.5 mg of Al^{+3}/mL.

Ten female mice of 5 to 6 weeks of age and of the Balb/c, haplotype H-2d,q per group were immunized by the intraperitoneal route. Three batches of the pentavalent vaccine, one batch of the placebo and the reference batch of the vaccine against hepatitis B, 07-0902, were studied.

Twenty eight days later, the mice were bled by retro-orbital puncture and the response of anti - HBsAg antibodies was assessed by ELISA, coating the plates with HBsAg (solid phase). The sample was incubated in the wells of the plate and then HBsAg conjugated with horseradish peroxidase was added. Ortho-phenylendiamine was used as the chromogenic substrate to develop the reaction. The ELISA plate was read at 492 nm.

The batch passed the test if the value of the relative potency was equal or higher than 0.5 compared to the potency of the reference vaccine.

Determination of immunogenicity of the PRP-T

Groups of five F1 rabbits were immunized by the subcutaneous route with doses of 0.5 mL of the pentavalent vaccine corresponding to 10 µg of PRP-T. At the same time, rabbits were immunized with a control vaccine against Hib (Vaxem Hib, Chiron S.p.a, batch 3204) and the negative control was an aluminum phosphate placebo; 0.5 mg/mL.

The rabbits were immunized on days 0 and 14 and they were bled at 21 days after the first dose. Blood was collected individually.

The antibody response against PRP-T was assessed through a specific non-competitive and indirect ELISA system. Coating was performed according to international recommendations: a capsular polysaccharide of the bacteria was conjugated covalently to human serum albumin (HbO-HA; NIBSC, England). The sample was included to form the HbO-HA+Ac anti-PRP-T complex. This complex was bound to the conjugated mouse anti-IgG marked with peroxi¬dase. Orthophenylendiamine was used as the chromogen and the reaction substrate was H_2O_2. A yel-low-orange color appeared in the antibody positive samples.

The percentage of animals showing seroconversion for each dose was calculated for each test sample; the batch passed the test when seroconversion was found in at least 50 % of the animals immunized per study group and the average titer for each group of rabbits immunized with the pentavalent vaccine had to be equal or higher than 800 IU/mL [25].

Results and discussion

Aluminum adjuvants have been shown to have a great ability to combine with a wide variety of antigens [26].

Despite progress in the generation of new adjuvants, aluminum hydroxide and aluminum phosphate continue to be the most widely used adjuvants in humans worldwide, due to their high degree of safety and, above all, low cost [27].

Selecting the appropriate adjuvant and guaranteeing the maximum adsorption of the antigen allows the interaction time between the antigen, the antigen presenting cells (APC) and the lymphocytes to be prolonged. This is mainly due to the size of less than 10 mm that the antigen acquires when adsorbed to the particle of the adjuvant gel or when the antigen assumes a new particulate nature that facilitates this process [28]. This is why it is so important to ensure the maximum possible adsorption of the antigen to the aluminum adjuvant. The adjuvant mechanism also encompasses stimulation of immunocompetent cells through complement activation, induction of eosinophilia at the injection site, and macrophage activation [29].

The results of the adsorption dynamics of diphtherial anatoxin at different times are shown in figure 1.

In figure 1 each column represents the values of two experiments. The initial concentration (time 0) for the vaccine is 50 Lf/mL.

The adsorption process of diphtherial anatoxin begins after 30 min and lasts until 2 h. Between the concentration values determined at 1 and 2 h there is a significant difference. From 2 and until 5 h the concentration of this anatoxin in the supernatant does not show significant differences. According to the results obtained in each of the sampling times, the optimal adsorption time for diphtherial anatoxin under these conditions is 2 h where 5 Lf/mL were detected in the supernatant, which corresponds to 90 % adsorption with respect to the antigen added to the formulation. This value is higher than the WHO recommendation for the adsorption of the anatoxins, whose value must be equal to or greater than 80 %.

The fact that 10 % of the diphtherial anatoxin added to the formulation is not adsorbed, may be due to the population heterogeneity of the molecules formed

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after the detoxification process, which undoubtedly depends on the composition of the initial solution in the which toxins are found [30]. These compounds range from peptones, typical of the means used in the fermentation to obtain the diphtherial and tetanus toxins (NZ-Amina A and NZ-Case TT), which are based on enzymatic digestions of casein [31]. The conventional anatoxins obtained from the supernatant of the culture medium carry a large number of contaminants that covalently bind to the anatoxins, which constitute unnecessary antigenic determinants. This produces a heterogeneous population of anatoxin molecules that could have different electrical charges that interfere with their adsorption to the aluminum hydroxide gel.

Tetanic anatoxin adsorption

The adsorption dynamics of the tetanic anatoxin to the aluminum hydroxide gel is shown in figure 2. Each column represents the result of two experiments.

The initial concentration (time 0) of tetanus toxoid in the vaccine is 20 Lf/mL. This value is not represented in the graph since the scale would be higher, affecting the height of the columns that are the object of interpretation.

After 30 min of the adsorption process, 8 Lf of tetanic anatoxin were detected per milliliter of supernatant. This value is significantly different when compared to that obtained at one hour, where the concentration detected in the supernatant was 2 Lf/mL (Figure 2). From this time on, and up to 5 hours of adsorption, there is no significant difference between the detected values.

According to the results obtained after 1 h of interaction between the gel and the anatoxin, the maximum adsorption is achieved; this corresponds to 90 % of the total added tetanus anatoxin to the aluminum hydroxide gel. This result is superior to that recommended by the WHO for the adsorption of this antigen, which must be equal to or greater than 80 %.

In this case, all the tetanic anatoxin is not adsorbed due to the heterogeneity of molecules obtained in the detoxification process.

In the results obtained from 1 to 5 h, a fluctuation in the concentration values between 2 and 3 Lf/mL is observed, this is mainly due to the variability of the method used, which is generally between 5 and 20 % [32].

Hepatitis B surface antigen adsorption

During the study of the adsorption dynamics of HBsAg, the results shown in figure 3 were obtained.

The initial concentration of HBsAg in the vaccine (time 0) corresponds to 20 µg/mL of the vaccine. This value is not represented in the graph since the scale would be higher, affecting the height of the columns that are the object of interpretation.

After 15 min, 8 µg of HBsAg were detected per milliliter of supernatant, this result is significantly different for $P < 0.05$ compared to 2 µg of HBsAg per milliliter detected at 30 min. This value corresponds to 90 % adsorption.

After 1 h, HBsAg is not detected in the supernatant, so it has been completely adsorbed.

The complete adsorption of this antigen to aluminum hydroxide is favored since, in its chemical composition, it has a lipid bilayer composed mainly of more than 70 % phospholipids. Their phosphate

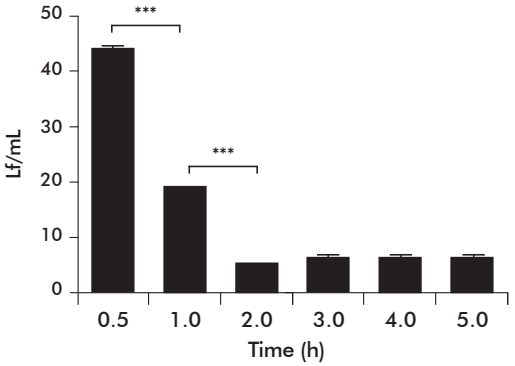

Figure 1. Diphtherial anatoxin concentration present in the corresponding vaccine supernatant at the indicated times. The statistical difference of the concentration values in the vaccine supernatant were calculated using a Bonferroni Multiple comparison test and are represented in the figure (*** $p < 0.001$). Error bars stand for the standard deviation of the mean (n = 3).

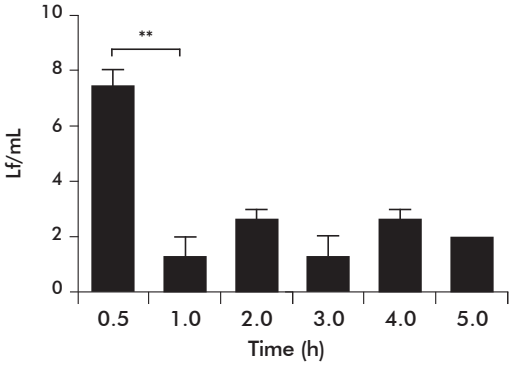

Figure 2. Adsorption dynamics of the tetanus toxoid concentration present in the corresponding vaccine supernatant at the indicated times. The statistical difference of the concentration values in the vaccine supernatant were calculated using a Bonferroni Multiple comparison test and are represented in the figure (** $p < 0.01$). Error bars stand for the standard deviation of the mean (n = 3).

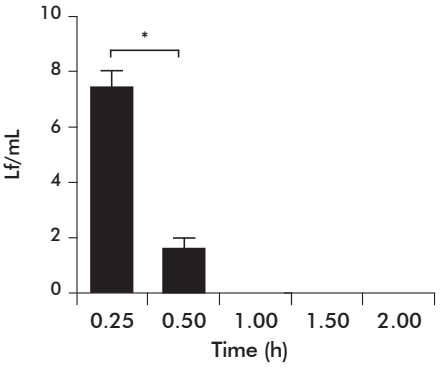

Figure 3. Adsorption dynamics of the hepatitis B surface antigen (HBsAg) concentration present in the corresponding vaccine supernatant at the indicated times. The statistical difference of the concentration values in the vaccine supernatant were calculated using a Bonferroni Multiple comparison test and are represented in the figure (* $p < 0.05$). Error bars stand for the standard deviation of the mean (n = 3).

groups strongly bind to the hydroxyl groups on the surface of the adjuvant by a ligand exchange mechanism, establishing a strong binding [33, 34]. The mechanism of adsorption of HBsAg to aluminum hydroxide has been studied by Iyer *et al*., showing the resistance of this antigen to the elution or separation of the gel when it is exposed to interstitial fluid [35].

Adsorption of poliribosyl ribitol phosphate conjugated to tetanus anatoxin

The aluminum phosphate adjuvant was selected for the adsorption of the PRP-T after mixing with the rest of the antigens in the pentavalent vaccine. The definition is supported by some antecedents published in the literature where it is demonstrated that poliribosyl ribitol phosphate (PRP) is a structural constituent of Hib, which presents a catalytic depolymerization in the presence of aluminum hydroxide gel. This phenomenon occurs over time as a consequence of a chemical reaction, in which the phosphate present in the PRP phosphodiester bonds compete with aluminum hydroxide specifically with the aluminum ion. Consequently, the said bond is susceptible to basic hydrolysis and there is a breakdown of the molecular structure of the polysaccharide which affects the stability of this antigen in the vaccine [36]. For this reason, this antigen was adsorbed to the aluminum phosphate gel independently before being added to the formulation to protect it from the action of aluminum hydroxide.

The results of the adsorption kinetics of the PRP-T are shown in figure 4. This process indicates that the largest amount of this antigen absorbs onto the aluminum phosphate at 2 h, representing approximately 42 % of the total PRP-T added. It is evident that a negative electrostatic effect is expressed, as occurring with the rest of the antigens with charges that are opposite to those of the gel, and, therefore, the predominating interactions are the hydrophobic interactions.

Adsorption experiments of the PRP-T onto the aluminum phosphate at pH 3 to 4 have been carried out, with percentages of adsorption above 97 %. But, when the pH was increased to reach a value that was near neutrality, more than half of the PRP-T in the vaccine de-adsorbed from the aluminum phosphate, reaching only 40 % of PRP-T adsorbed. This event was caused by the increase of the H^+ ion concentration of HCl, since this ion is small and can readily bind to the negative surface charges of the gel, thereby inverting the net load of the aluminum phosphate from negative to positive. Hence, the electrostatic interaction between the PRP-T and the aluminum phosphate is facilitated. In this process of increasing the vaccine pH up to 6.85, 0.2 M NaOH was added, the OH^- ions displacing by size the PRP-T previously adsorbed. Therefore, this antigen becomes de-adsorbed as shown in figure 5.

Adsorption of whole cells *B. pertussis*

Whole cells of *B. pertussis* present in the cell wall have a chemical composition based mainly on proteins and lipids. Some of these compounds favor adsorption to aluminum hydroxide, however the size of these cells intervenes in the slow process of adsorption to the adjuvant [37].

The initial concentration (time 0) of *B. pertussis* cells in the vaccine is 32 OU/mL.

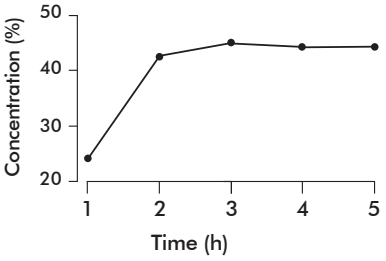

Figure 4. *Haemophilus influenzae* type b conjugated to tetanic toxoid (PRP-T) concentration present in the corresponding vaccine supernatant with the indicated times.

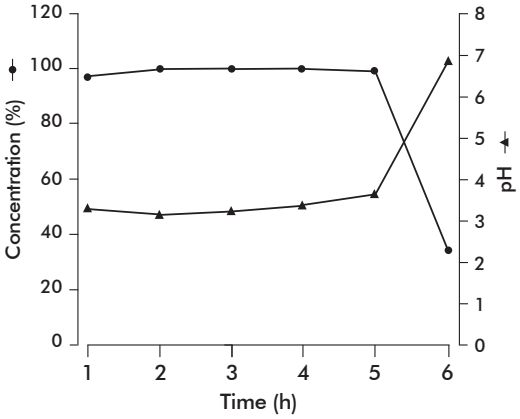

Figure 5. *Haemophilus influenzae* type b conjugated to tetanic toxoid (PRP-T) concentration present in the corresponding vaccine supernatant with the indicated times, corresponding to adsorption percentage of PRP-T to aluminum phosphate and pH, respectively. Time refers to the moment the samples were collected.

The adsorption process of these cells occurs slowly as can be seen in figure 6. After 3 h, the adsorption process has not started since the total added cells are detected in the supernatant. This process starts at 6 hours and the values obtained are significantly different compared to those obtained at 9 and until 12 h (Figure 6). From 12 and until 30 h, there is no significant difference in the concentration of cells in the vaccine supernatant. However, it is important to point out that at 12 h there were attained 8 OU/mL in the supernatant of the formulation and from 18 to 30 h between 5 and 6 UO/mL were detected. Therefore, this means 2 to 3 billion more cells per milliliter of vaccine.

These results, represented in percent adsorption, show a difference, for example, at 12 h, 75 % of the total pertussis cells added to the formulation are adsorbed, but after 18 h, 81 % adsorption is reached. Although there were no significant differences between the concentration of adsorbed cells between 12 and 18 h, this last period was selected as the optimal time for the adsorption of whole *B. pertussis* cells. This was supported by the higher percentage of adsorption that could influence the immune response of this component, observed from that time onwards.

Physicochemical and biological evaluations of the three lots of pentavalent vaccine

Table 2 shows the physicochemical and biological results of the 3 formulated batches of the DPT-HB-

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Hib pentavalent vaccine as well as the specifications established for this stage.

As shown, the three batches met all the specifications established for the release of this product.

The organoleptic characteristics did not show any change or difference between the batches, the resuspension of the vaccine was carried out in a simple way and the color remained identical for the three batches.

The pH was around neutrality between 6.73 and 7.0, which reduces the possible adverse events of this product when it is inoculated trying to reach a pH very similar to the interstitial liquid. Furthermore, these values guarantee that this parameter does not influence the physico-chemical characteristics of antigens.

Both the production process, production areas and preparation of materials as well as the closure system of the vials guarantee the sterility of the product that makes it optimal for use. This was demonstrated with the satisfactory sterility results for all three batches.

Aluminum content is an important aspect, as it can be seen in the results they were between 0.58 and 0.68 mg Al3+ per mL of vaccine, trying to achieve a compromise between the adsorption of antigens and a minimal reactogenicity of the product when administered. As it is known, the adjuvants of aluminum salts produce indurations and pain when inoculated.

The thiomersal preservative plays a very important role in the product in maintaining the growth of microorganisms. The content found in the controls of the vaccine lots was within the specification range for this parameter.

The tests for specific toxicity and general safety were successfully passed, which shows that the mixture of all the elements that are part of the vaccine as well as their content make up a non-toxic product.

The five antigens present in the vaccine are identified, and also show an adequate biological response that is expressed in the potency and immunogenicity results for each antigen.

The physicochemical and biological results of these batches gave us the possibility of having a technology that was the basis for the development of the Cuban pentavalent vaccine Heberpenta®-L in all its subsequent stages.

Conclusions

It is a technological challenge to have a pentavalent vaccine for all the problems that may appear, such as negative interactions between antigens that would affect the quality of the vaccine, the extensive and necessary quality control, as well as productive assurance.

The results of this study have shown to be positive, which allowed us to continue advancing in the development of a Cuban pentavalent vaccine, with the execution of later stages such as non-clinical, stability and clinical studies, which will be presented later in other publications.

There are few vaccine companies that have a liquid pentavalent that includes these five antigens; therefore achieving a national vaccine not only for use in Cuba but in other countries is very important. On the other hand, this development facilitates collaboration with other laboratories of the world in response to the WHO call for the development of combined vaccines due to their shortage.

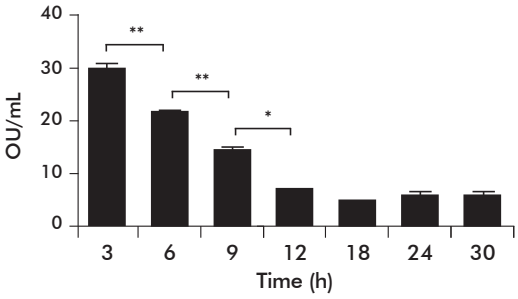

Figure 6. *Bordetella pertussis* cell concentration present in the corresponding vaccine supernatant with the indicated times. The statistical difference of the concentration values in the vaccine supernatant were calculated using a Bonferroni's multiple comparison test and are presented (*$p < 0.05$, **$p < 0.01$). OU: opacity units. Error bars stand for the standard deviation of the mean (n = 3).

Table 2. Batches of active pharmaceutical ingredients (API) used in the formulation of the Cuban pentavalent vaccine

Assay	Specifications	Vaccine batches		
		EPL0301E	EPL0302E	EPL0401E
Organoleptic properties	Slightly opaque, particle-free white suspension	Pass the test	Pass the test	Pass the test
pH determination	6.4-7.7	6.79	6.73	7.00
Sterility	Satisfactory	Pass the test	Pass the test	Pass the test
Aluminum content	0.3-0.85 mg/mL	0.63	0.68	0.58
Thimerosal content	0.035-0.1 mg/mL	0.065	0.071	0.065
General safety	No symptoms or deaths in 21 days	According	According	According
Specific toxicity of diphtherial and tetanus anatoxins	No symptoms or deaths in 6 days	According	According	According
Bordetella pertussis toxicity	60 % increase in weight of animals	According	According	According
Identity of diphtherial anatoxin	40-60 Lf/mL	50	50	50
Identity of tetanic anatoxin	16-24 Lf/mL	20	20	20
Identity of *Bordetella pertussis*	Agglutination positive	Agglutinate	Agglutinate	Agglutinate
Identity of HBsAg	Positive by ELISA	Identified	Identified	Identified
Identity of PRP-T	Agglutination positive	Agglutinate	Agglutinate	Agglutinate
Potency of diphtherial anatoxin	≥ 2 IU/mL	2.51	2.51	2.72
Potency of tetanic anatoxin	≥ 2 IU/mL	5.30	7.30	5.85
Potency of *Bordetella pertussis*	≥ 4 IU/ 0.5 mL	5.1	6.1	4.3
Potency *in vivo* of HBsAg	≥ 0.5	1.5	2.5	1.6
Inmunogenicity of PRP-T	≥ 50 % of the animals	100 %	100 %	100 %

HBsAg: Hepatitis B virus surface antigen. PRP-T: *Haemophilus influenzae* type b conjugated to tetanic toxoid

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