Development of a immunochromatographic test with avidin-biotin for the detection of antibodies against antigen e of hepatitis B in human plasma

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ABSTRACT

The disappearance of antigen e of hepatitis B in the presence of the plasmatic antibodies against antigen e may indicate a satisfactory therapeutic response in patients with chronic hepatitis B. The immunochromatographic test carried out in the diagnosis of diseases use different antibody combinations and may employ the avidin or streptavidin-biotin technology to develop a rapid immunochromatographic test for the detection of antibodies anti-antigen e in the plasma. They were detected in the laboratory by means of two fast immunochromatographic tests when using in one of them the avidin-biotin technology. These tests are carried out with a one-step competitive inhibition format and amplified or not with avidin-biotin. Monoclonal antibodies against antigen e obtained by cellular hybridization were used. Forty-six plasmatic samples classified as positive and negative to the anti-antigen antibodies were evaluated with a reference immunochromatographic test Advanced Quality™. The possible expiry time of the biological reagents forming part of these tests were studied with accelerated thermal-stability experiments. The possible interference in the plasma of some of the biochemical compounds used in these trials was analyzed. Four murine monoclonal antibodies anti-antigen e were obtained and only one of them was used in these immunochromatographic tests with an anti-antigen polyclonal antibody conjugated with gold. Both tests and their stable biological reagents discriminated the positive and negative samples to the antibodies anti-antigen e, as well as the commercial test. There was no interference in the biochemical compounds studied in these tests. Both immunochromatographic tests made in the laboratory are useful to detect antibodies anti-antigen e in the plasma. The avidin-biotin increased the analytical sensitivity of this type of fast immunochromatographic test without altering its performance features.

Keywords: Immunochromatographic analysis, avidin-biotin, colloidal gold, hepatitis B, diagnosis

Introduction

Viral hepatitis B (HB) is a worldwide problem that affects about 5% of the world population [1]. It is the prime cause of cirrhosis and hepato-cellular carcinoma. The natural protein of antigen e of HB (HBeAg) is originated from one part of the gene of the core antigen of HB (HBcAg) and it shares a great homology in the amino acid sequence with AgeHB. These antigens are however recognized by natural antibodies against...
different epitopes of both macromolecules [2]. The disappearance of HBeAg with the presence of anti-HBeAg antibodies in the plasma is an indicator of a good prognosis and of an appropriate anti-viral therapeutic response of patients with an acute or chronic wild type hepatitis B virus (HBV) infection [2, 3]. Also the presence for years of these antibodies in patients with negative diagnostic tests to HBeAg indicates a chronic infection with mutants of the pre-coregenic region of HBV VHB [1] and the prognosis of these patients is unfavorable.

The immunochromatographic trials offer a fast way of performing the analytical procedures and also a way of carrying out diagnostic tests with the least possible use of reagents. These analyses can be performed in the area where the biological samples are collected, and not necessarily in the places where there is specialized personnel and equipment. Immunochromatographic trials have been described to detect antibodies or antigens that can be competitive or non-competitive [4]. In the non-competitive trials it is found that the higher the amount of the analyte in the sample, the greater the staining in the strip. In these trials a positive or negative response depends on the presence or absence of the analyte, respectively. While in the competitive analyses the opposite occurs, a larger amount of the analyte in the sample makes the sign disappear more rapidly. In the competitive immunoanalyses a positive sample is that when there is no sign on the strip, and a negative sample is when the strip is stained [4].

In these fast one-step immunoassays based on membranes with lateral flow the avidin or streptavadin-biotin technology has been used to increase analytic sensitivity [5-7]. The streptavidin or avidin has been placed in the specific capture zone of the nitrocellulose membrane and two antibodies that detect two different sites of the substance were placed on the cushion of the sample [5]. One of those antibodies is biotinilated and the other is conjugated with gold. These two antibodies react for more time with the analyte to detect the biological sample, and form an antigen-antibody complex that will be much better trapped by the streptavidin in the specific capture zone of the test in a short time.

In the trials described we use the following characteristics of the interaction between the streptavidin or the avidin by the biotin: - Being on of the strongest “non-covalent” links known today (the affinity constant of this link is of $10^{15}$ M$^{-1}$). - Being a highly specific bond, in the avidin and the streptavidin there are 4 binding sites for the biotin, when adding to it (a small molecule of 244 Da) a space arm it can conjugate with the antibodies without altering its biological activity or that of the macromolecule.

- The avidin and streptavidin show a good stability under different reaction conditions and immobilization in solid phases without affecting the binding sites with the biotin [8].

- There are differences between the streptavidin and the avidin; one of these is given by the isoelectric point. In the first case it ranges from 6.4 to 6.6 and in the second it varies from 10 to 10.6. The other aspect is that the streptavidin does not have carbohydrate molecules in their structure, and avidin is a glycoprotein [8, 9]. The avidin used in the immunonanalysis with the lack of specificity difficulties can be substituted by streptavidin because it does not have a strong positive charge in the tests made at neutral pH. Its interaction with molecules that are negatively charged is decreased and it is not trapped by the molecules that bind carbohydrates (e.g., the lectins of the cell surface) of the biological samples, above all in immunochemical techniques.

In the market there are immunochromatographic trials for the detection of the anti-HBeAg response that contains two groups of antibodies: one is conjugated with gold in the macroporous cushion, and the other is coated in the nitrocellulose. Both antibodies recognize the HBeAg through 2 sites and may be inhibited competitively by the presence of antibodies in the biological sample [10, 11]. The detection of the anti-HBeAg antibodies in the plasma using the avidin or streptavidin-biotin technology in an immunochromatographic competitive inhibition test has not been reported.

In this paper we describe two immunochromatographic tests for the detection of plasmatic anti-HBeAg antibodies, in which monoclonal antibodies (MAb) anti-recombinant HBeAg (anti-rHBeAg) generated in the laboratory are used. An immunochromatographic test applies the first format stated above, and the other adds the use of avidin-biotin to increase the sensitivity for the detection of these antibodies. Both immunochromatographic trials are standardized and studies on the accelerated stability of its biological reagents are carried out. With these two tests made in the laboratory, we evaluated 46 plasmatic samples classified as positive and negative, according to the present of antibodies anti-HBeAg through a reference immunochromatographic test Advanced QualityTM (InTec Products Inc., Xiamen, China).

Materials and methods

Generation and MAb anti-recombinant AgeHB

The monoclonal antibodies were obtained as described before [12, 13]. In short: female BALBc mice were immunized with 50 μg recombinant HBeAg (HBeAg, SD Standard Diagnostic Inc., Korea) mixed in the same volume of complete adjuvant the first time, and then incomplete Freund adjuvant (Sigma, Saint Louis, USA) and injected to mice by the subcutaneous route. The final immunization of the antigen was made by the intraperitoneal route with the same amount indicated dissolved in the saline solution of phosphate pH 7.2 at 3 days before the fusion. The hybridoma were obtained by the fusion with polyethylene glycol 1600 (Sigma, St Louis, USA) of the myeloma P3X63-Ag8-653 and the splenic lymphocytes of the immunized mice in a ration of 10 to 1. The positive cultures selected in an enzymatic immunonalysis (described later) were cloned twice by the limiting dilution method. To determine the subclass


of the selected MAb the manufacturer’s instructions were followed (Amersham, United Kingdom) of a commercial case of isotypification of mice MAb in the supernatant of the cell culture.

The selected hybridoma grew as ascitic tumors in BALBc mice that had been inoculated intra-peritoneally with 2, 6, 10, 14 tetramethylpentadecane (Pristane of Sigma, St Louis, USA) and the antibodies of the ascitis were purified by affinity chromatography using protein A searose CL 4B (Pharmacia Biotech, Uppsala, Sweden). Its purity was assessed by 15% polyacrylamide gel electrophoresis with dodecyal sodium sulphate [9]. The aliquots were stored at -20 °C. The purified antibodies were conjugated to NHS-LC-biotin (Sigma, St Louis, USA) according to Perlman [14].

Immunoenzymatic assay for the detection of the anti-rHBeAg antibodies

An indirect immunoenzymatic assay was used for the determination of the anti-rHBeAg antibodies in the sera of immunized mice, supernatants of cultures and purified antibodies as described in other papers [12, 13]. Briefly, the polystyrene micro-titration plates with 96 wells of a flat bottom Maxisorp (Nunc, Roskilde, Denmark) were coated with 50 µL/well of rHBeAg at 20 µg/mL in sodium bicarbonate-carbonate (Merck; Darmstadt, Germany) 50 mM pH 9.6 for 16 h at 4 °C. After washing and blocking the plates the different dilutions of the sera of immunized mice with the antigen (positive control) were added and the negative control used were the dilutions of the supernatant of the culture of MAB anti-surface antigen of HB CBHep.1 (CIGB, City of Havana, Cuba) and 1 µg/mL of rHBeAg and applied in 2 independent wells in a final volume of 50 µL/well, and so on with another two antigens in the same concentrations: rHBcAg and human serum albumin (Sigma, St Louis, USA). In this step we also included a duplicate of the wells for each one of the dilutions of the supernatants of the hybridoma culture where we did not add any other protein. The trial continued as in the previous section. The calculation of the percentage of inhibition was made with the following formula: % inhibition = (abs c+ -abs m)/(abs c+ -abs c-))*100%, where abs m corresponds to the mean of the duplicate of the absorbances of the supernatant of the culture of a hybridoma with anti-rHBeAg antibodies to which we added the antigen at the dilution, abs c- is the mean of the duplicate of the absorbances of the supernatant of the culture with antibodies of a specificity different from the antigen added and abs c+ is the mean of the duplicate of the absorbances of the supernatant of cultures of the same hybridoma in the analysis without adding antigen to the dilution. It was considered that there was inhibition when it was over 20%. In this percentage we included the mean plus two standard deviations of the inhibition that existed in some supernatants of the culture of the hybridoma studied when human serum albumin was added.

Principle of the immunochromatographic tests amplified or not with avidin-biotin

The nitrocellulose strip with a plastic back support containing a coat of glue (with a pore of 10 µm and a size of 86 mm x 5 mm), the absorbent cushion (38 mm x 5 mm) and the macroporous cushion (with a pore of 10 µm and a size of 26 mm x 5 mm) -acquired from Advanced Microdevices, Ltd, Ambala, India- constituted the solid phase of both tests (Figure 1a). The surfactants: Pluronic™ and Igepal™ and the polymers Kucell™ and polyethylene glycol or PEG 8 000 (commercial case from Pragmatics Inc., Indiana, USA) were used as blocking agents of unspecific reactions and stabilizing additives in these tests. We applied 150 ng of the poly-L-lysine (Sigma, St Louis, USA) in the area for the control of the functioning of the strip of nitrocellulose of the two immunochromatographic tests (that is found at approximately 20 mm from the lower edge of the nitro-cellulose membrane) and the MAB anti-rHBeAg, in the specific capture zone of the non-amplified test (Figure 1b). The latter zone is found at approximately 15 mm from the lower edge of the nitrocellulose membrane and
we applied on it the avidin (Sigma, St Louis, USA) in the amplified test (Figure 1c). In both tests we used a combination of the two antibodies: one was the MAb anti-rHBeAg and the other was the anti-rHBeAg polyclonal antibody (PAb) in mice, conjugated with colloidal gold particles (PAb-gold). The commercial conjugate of PAb-gold (SD Standard Diagnostic, Kionggi-do, Korea) that has an optical density of 10 to 540 nm (with a diameter of the gold particle of 40 nm) was applied at the upper end of the macroporous cushion. In the two test the rHBeAg was poured at different concentrations (according to the experiment) at 5 mm of the lower edge of the macroporous cushion. This antigen was added simultaneously with the MAb anti-rHBeAg biotinylated in the amplified test.

After the reagents applied on the nitrocellulose membrane and on the macroporous cushion are dried, we set up the macroporous cushion on the lower part of the immunochromatographic strip and the absorbent cushion on the upper part. The upper edge of the macroporous cushion and the lower edge of the absorbent cushion are overlapped with the nitrocellulose membrane of immunochromatographic strip. The immunochromatographic tests start when the nitrocellulose strip with all its elements set up is introduced by the lower part of the macroporous cushion in a well of an inert plastic plate of 96 wells and a flat bottom (Polylabo, Paris, France) containing 100 µL of human plasma (Figure 1a, 1b and 1c). This sample of the plasma will start migrating by the capillarity of the strip. Both tests were withdrawn from the corresponding wells after running the immunochromatographic test for 20 minutes at room temperature (from 20 to 25 °C) and the result was read at that time (Figure 1d). A negative result was that when there were two visible stains in the reaction area. The positive result was if there was only one stain corresponding to the control of the test and a non-valid test was considered when there was no sign. In the latter case, the test was repeated. The visual reading of the results of the two immunochromatographic tests was made with an appropriate illumination.

In the non-amplified test where we evaluated a sample without the antibody anti-HBeAg, the HBeAg applied on the macroporous cushion was transferred by the plasma until the end of the macroporous cushion and was bound to AcP-gold. Later this antigen-antibody complex reaches the capture zone where it is trapped by the immobilized MAb anti-rHBeAg. The concentration of the gold particles of this immunological complex gives way to a purple red stain in that region. When the migration continues in the front of the run, the antibody conjugated with gold that did not react with the antigen was trapped in a second unspecific capture zone where the poly-L-lysine charged positively is found and interacts with the negative charges of the gold particles. At that time there was a second signal that was originated and worked as a control for the migration of the PAb-gold in the biological matrix through the specific capture line (Figure 1b and 1d). In the case of having anti-HBeAg antibodies in the plasma, these combine with the rHBeAg and inhibit firstly the bonds of this antigen with the PAb-gold in the macroporous cushion and later, with the MAb present in the capture line of the nitrocellulose.

In the amplified test with avidin-biotin, the plasma sample without the antibodies anti-HBeAg dragged the rHBeAg with the biotinylated MAb applied on the macroporous cushion up to the site where the PAb-gold was dried. In this path there was an interaction
between these reagents. Then this complex was trapped in the specific capture zone of the nitrocellulose with the immobilized avidin and produced a stain, the excess PAb-gold was bound later by the poly-L-lysine and there was a second sign. This situation corresponds to the presence of anti-HBeAg antibodies in a negative sample. In the case of a positive sample for these antibodies, there was only one signal because the rHBeAg was recognized by them and blocked the bond of the biotinylated antibody and the PAb-gold conjugate with this antigen, for which reason the complex was not formed, which would have been trapped by the immobilized avidin in the nitrocellulose (Figure 1c and 1d).

Optimization of the non-amplified immunochromatographic test

The MAb anti-HBeAg solution was applied in a volume of 2 µL in the nitrocellulose at different amounts: 0.4, 0.8, 1.8, 3.5, and 7 µg in a diluent containing trehalose (Sigma, St Louis, USA) 5% (w/v) and Igepal 0.05% (v/v) of the final concentration. The MAb anti-HBeAg control solution corresponded to a previous purified antibody batch. We poured 1 µL of 150 ng of poly-L-lysine diluted in a 10% trehalose solution within the area used for the control of the functioning of the test. The strip of nitrocellulose was incubated at 37 °C for 16 h. The solutions of PAb-gold and rHBeAg were diluted 1/2 and 1/5, respectively, in a stock blocking and thermostabilizing solution formed by 20 mM Tris, pH 8.5, with 20% (w/v) bovine serum albumin, 10% (w/v) trehalose and 0.1% (v/v) pluronic. We applied 2 µL of both diluted solutions on the macroporous cushion in both sites mentioned previously and incubated the cushion at 37 °C for 1 h. The final amount of rHBeAg added was 80 ng. Later the macroporous cushion and the absorbent cushion were set up on the nitrocellulose strip. Each coating condition of the MAb anti-rHBeAg was analyzed with a sample of the negative plasma and another positive one for anti-HBeAg antibodies (Figure 2).

The PAb-gold was optimized by diluting it at 50% in the above mentioned diluent and applying 1 µL, 2 µL, 3 µL, 4 µL, 5 µL and 6 µL on the upper edge of the macroporous cushion. It was later incubated for 1 h at 37 °C. In each condition of the conjugate a plasma sample was tested with the anti-HBeAg antibodies and without them. The coating of the nitrocellulose with the anti-rHBeAg MAb was of 1.8 µg per immunochromatographic strip. The coating conditions of the poly-L-lysine in the nitrocellulose and the application of 80 ng of rHBeAg in the macroporous material were similar to those mentioned in the above paragraph.

Different amounts of rHBeAg to be neutralized in the macroporous cushion were tested: 100 ng, 80 ng, 60 ng, 40 ng and 20 ng diluted 1/5 in the stock blocking and thermostabilizing buffer. In those macroporous cushions at the two sites we applied a final volume of 2 µL of the solutions of rHBeAg and 2.5 µL PAb-gold. The cushions were incubated at 37 °C for 1 h to dry the biological reagents. The coating of the nitrocellulose with 1.8 µg of MAb anti-rHBeAg and 150 ng of poly-L-lysine was performed as described earlier. For each conditions of the rHBeAg we evaluated a sample of the positive plasma and another negative one to the antibodies anti-HBeAg.

Optimization of the amplified immunochromatographic test

The strips of nitrocellulose were coated at the specific capture zone with 2 µL (2 µg) of an avidin solution (Sigma, St Louis, USA) at 10 mg/mL diluted 1/10 under two conditions: in distilled water with a final pH of between 3 and 4 (pH paper from BDH, England, United Kingdom) and in a buffer of bicarbonate-carbonate 10 mM pH 9.6 (with a final pH of between 9-10 in the pH paper of BDH). Also the nitrocellulose was coated at the control zone for the functioning of the test with a solution of poly-L-lysine. Then the strips were dried at 37 °C for 16 h. The solutions of PAb-gold and rHBeAg with the biotinylated MAb anti-rHBeAg were applied on the macroporous cushion with a blocking and thermostabilizing mother solution (which is the same one used in the non-amplified immunochromatographic test) diluted 1/2 and 1/5, respectively. We applied 2 µL of the diluted solutions of PAb-gold and of the joint dilution of HBe Ag (80 ng) plus the biotinylated anti-rHBeAg MAb (400 ng) in the macroporous material. For the negative control we poured 2 µL of 80 ng of rHBeAg plus 400 ng of the non-biotinylated anti-rHBeAg MAb on that cushion. The conjugate and the antigen with the biotinylated and non-biotinylated MAb were dried at 37 °C for 1 h. Then the macroporous cushion and the absorbent cushion were set on the nitrocellulose strip. The tests were introduced in 100 µL well of a sample of negative plasma to the anti-HBeAg antibodies.

Different relations of the mass of rHBeAg and the biotinylated anti-rHBeAg MAb were tested in order to decrease the amount of rHBeAg to be neutralized in the amplified immunochromatographic test. We applied 2 µL of 80 ng of rHBeAg with 400 ng of biotinylated MAb and 40 ng of the former with 400 ng of the latter. Afterwards the amounts continued as:
The comparison of the two immunochromatographic tests with the commercial immunochromatographic test for the detection of the anti-HBeAg antibodies

A group of 46 samples of plasma with antibodies anti-HBeAg and without them were evaluated in an immunochromatographic test Advanced Quality™ for the detection of antibodies anti-HBeAg. This commercial immunochromatographic assay was performed according to the manufacturer’s instructions (it had a high equivalence in the detection of anti-HBeAg antibodies in 1000 samples in relation to an enzymatic immunoanalysis) [10]. The possible results in an upper view of that commercial test are similar to the readings carried out on the tests made in the laboratory (Figure 1d). Later, the samples analyzed in the commercial reference test were studied with the two immunochromatographic tests from the laboratory.

Interference test of the anti-HBcAg antibodies, biotin, interferon alpha 2b and heparin in the immunochromatographic tests

A group of 5 plasmatic samples were negative to the antibodies anti-HBeAg and positive to the anti-HBeAg antibodies in a one-step multi-immunochromatographic assay (Multi-HBV) from Advanced Quality™ (InTec Products, Inc., Xiamen, China). This commercial assay that determines these 2 types of antibodies, besides another 3 markers related to the diagnostic of HB was carried out according to the manufacturer’s instructions. This group of 5 samples was evaluated with the two immunochromatographic tests from the laboratory to verify that there was no interference of these antibodies anti-HBcAg in the determination of anti-HBeAg antibodies.

Processing the samples

The samples used belong to the bank of 0.8% (w/v) sodium citrate-plasma and plasma-EDTA (2 mg/mL of disodic ethylen-diamine-tetracetic salt) of anonymous positive and negative samples to the HB surface antigen or to the antibodies anti-HBcAg of the Immunodiagnostic Department stored at -20 °C. We thawed them and they were centrifuged at 4 000 rpm for 15 min at room temperature before using them to eliminate the remains of fibrin. Aliquots were prepared so as to not increase to more than 3 cycles the thawing and freezing process. The hemolytic or very thick plasma with the fibrin remains were not analyzed.

Thermostability test of the compounds of the immunochromatographic tests

The main components of the immunoanalyses, the anti-rHBeAg MAb, the PAb-gold, rHBeAg, the biotinylated anti-rHBeAg MAb and the avidin were assayed in two concentrations of trehalose with other additives to evaluate stability at 60 °C at 3 and 5 days, compared to when the temperature was at 20 to 25 °C. A sample was used as the positive plasma and the other one as negative plasma to anti-HBeAg antibodies. The volume of the solution of the solution of poly-L-lysine (150 µg/mL) diluted in trehalose at 10% used in all experiments was of 1 µL. In the immunochromatographic test without amplification, the MAb was absorbed to the nitrocellulose with a solution of isepal 0.05% and trehalose at 4% and 8% and the PAb-gold conjugate was applied on the macroporous material diluted to 50% in the blocking and thermostabilizing buffer in

Figure 3. Determination of the optimum relation of recombinant antigen α of the Hepatitis B virus (rHBeAg) and a biotinylated monoclonal anti-rHBeAg antibody (MAB B) CBHeP8.AgE (MAB B) in the macroporous cushion of the amplified immunochromatographic test to detect anti-HBeAg antibodies. The immunochromatographic test 10 corresponds to the proportion 400 ng of non-biotinylated MAb CBHeP8.AgE and 80 ng of rHBeAg.

The biotin (BDH, England, United Kingdom) and the recombinant interferon alpha 2b (CIGB, City of Havana, Cuba) were added to 20 ng/mL and 100 ng/mL of each one of them in a plasma negative to, and another one positive to antibodies anti-HBeAg. The negative controls included were the plasma that were positive and negative without adding these reagents. All samples were run in the immunochromatographic tests developed in the laboratory.

The interference of the heparin (Imefa, City of Havana, Cuba) was studied in the non-amplified and the amplified immunochromatographic test. In both of them we tested three concentrations of heparin: 0 IU/mL, 2 IU/mL and 10 IU/mL, in samples of plasma with and without anti-HBeAg antibodies. The poly-L-lysine was coated at 150 ng in the two types of tests. In each strip of nitrocellulose of those 12 immunochromatographic tests of each type, we set up a macroporous cushion that had been applied and left to dry at the upper edge with 2.5 µL of PAb-gold diluted previously with the thermostabilizing and blocking buffer at 50%. Later these two types of tests were repeated with the application of 2 µg (2 µL) of anti-immunoglobulin G from mice in sheep (CIGB of Sancti Spiritus, Cuba) dried for 1 h at 37 °C, instead of poly-L-lysine; in the zone for the control of the functioning of the test. Later these tests were introduced in the wells containing the sample of positive and negative plasma for the antibodies anti-HBeAg with the different concentrations of heparin expressed previously.

Figure 3. Determination of the optimum relation of recombinant antigen α of the Hepatitis B virus (rHBeAg) and a biotinylated monoclonal anti-rHBeAg antibody (MAB B) CBHeP8.AgE (MAB B) in the macroporous cushion of the amplified immunochromatographic test to detect anti-HBeAg antibodies. The immunochromatographic test 10 corresponds to the proportion 400 ng of non-biotinylated MAb CBHeP8.AgE and 80 ng of rHBeAg.
two final concentrations of the trehalose: 10% and 20%. In the amplified immunochromatographic test 2 µg de la avidin, diluted in bicarbonate-carbonate 10 mM pH 9.6 without trehalose and with trehalose at 5% and at 10%, was coated on the nitrocellulose strip. The proportion of 200 ng of the biotinilated MAb with 40 ng rHBeAg was applied on the macroporous cushion in its diluent 1/5 with trehalose at 4% and at 8%.

We briefly explain the other steps for the storage of these tests [15]. The immunochromatographic strips and the macroporous cushions with the different biological reagents and their unspecific stabilizing and blocking additives were introduced separately in different nylon metal containers with a desiccant silica gel bag Minipax™ (Multiform Desiccants Inc., New York, USA) inside. Later these containers were sealed. Afterwards a group of strips ans another group of cushions sealed in nylon were placed in an oven Yamato Scientific, Co. Ltd, Japan) at 60 °C and another group of them were kept at 20 to 25 °C. The different components of the immunochromatographic tests in the stability study (both amplified and without amplification) were assembled with the remaining parts of this type of test that contains the biological reagents prepared and added on the same day. In all cases we proved the functioning of the immunochromatographic one step tests in 100 µL of plasma with antibodies anti-HBeAg or without them in a run of 20 min at room temperature.

Statistical analysis

The optimization experiments of the amplified and non-amplified immunochromatographic tests were made at least two separate times. The visual readings of both tests were carried out by at least two independent observers, without knowing previously the diagnostic of the sample. In the comparison of the immunochromatographic tests from the laboratory with the commercial test we used the determination of the kappa of Cohen statistics for the binomial variables with the aim of demonstrating if there is a relation or agreement between the two methods that detect one same analyte in the dependent samples. This calculation was carried out using Microsoft Excel (Microsoft Corporation, USA).

Results and discussion

Generation of the anti-rHBeAg MAb CBJHBpB.AgE

We used two lymphocyte fusion experiments with immunized animals, having titers of antibodies anti-rHBeAg greater than 1/1000 with mice myeloma. There were 4 hybridoma that secreted mice antibodies of class IgG. None of the antibodies recognized the recombinant surface antigen of HB. However, all of them were 4 hybridoma that secreted mice antibodies of rHBeAg greater than 1/1000 with mice myeloma. The MAb secreted by the hybridoma 242/27/6 worked similarly to the commercial anti-rHBeAg antibodies. We discriminated the two groups of antibodies secreted by the hybridomas: antibodies that recognized the rHBeAg 2 or 3 times less than the rHBeAg in solution and another group of antibodies that recognized the rHBeAg 2 or 3 times more than the rHBeAg in solution. The selection of the anti-rHBeAg MAb that would be included in the immunochromatographic tests for competitive inhibition to detect the antibodies anti-HBeAg was made by the way the rHBeAg in solution is recognized in respect to that of the absorbed antigen in the solid phase. The MAb secreted by the hybridoma 242/27/6 worked similarly to the commercial anti-HBeAg MAb in this last group of antibodies on recognizing better the rHBe Ab than the rHBeAg in solution, and it was selected as the capture antibody in the immunochromatographic test. The subclass of MAb secreted by this hybridoma was IgG2b. With this purified MAb we carried out the later experiments of the two types of immunochromatographic tests and it was named CBHepB.AgE.

Optimization of the non-amplified immunochromatographic test

The saturation of the red signal in the specific capture zone reaches 1.8 µg of the CBHepB.AgE and the intensity of the stain in the zone of the control of the functioning of the test does not decrease its coloration. We obtained a good discrimination of the red signal of the negative sample in relation to the absence of the signal in the positive sample, for which reason that was the amount of capture MAb used in the other experiments of the immunochromatographic test without amplification (Figure 2).

When we apply 1 µL of the volume of PAb-gold diluted at 50%, in the stock blocking and thmosatibulating buffer we obtain a weak red signal in the negative sample. After applying 2 µL of the volume of PAb-gold we obtained the saturation of the signal of the negative sample and perceived the inhibition of the red signal in the capture line by the positive sample. We also found at that volume of the conjugate, a good

Table 1: Recognition of the recombinant hepatitis B virus e (rHBeAg) and core (rHBcAg) antigens in solution, or absorption in the solid phase by antibodies secreted by hybridoma obtained in the laboratory and a commercial anti-HBeAg monoclonal antibody (MAb) in an indirect immunoenzymatic assay for detection of anti-HBeAg antibodies

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Intensity of the absorbance when the antigen is in the solid phase*</th>
<th>Percentage of inhibition of the absorbance by the competing antigen in solution**</th>
<th>Groups</th>
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<tr>
<td></td>
<td>rHBeAg</td>
<td>rHBcAg</td>
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<td>242/27/6</td>
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<tr>
<td>Commercial MAb</td>
<td>x</td>
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*Represented by the absorbance of 0 to 0.6 (x); of >0.6 to 1.2 (x); of >1.2 to 1.8 (xx); of >1.8 to 2.4 (xxx) and of >2.4 to 3 (xxxx).

**The inhibition percentage of 1 µg/mL of the recombinant antigen in solutions of 0% to 20% (x); of >20% to 40% (x); of >40% to 60% (xx); of >60% to 80% (xxx) and of >80% to 100% (xxxx) are shown.


sign in the control zone of the functioning of the test, and a greater contrast of the specific red sign with the more white background of the nitrocellulose. In the rest of the experiments we used between 2 and 2.5 µL of this reagent to ensure the saturation of the sign, both in the amplified immunochromatographic test and in the non-amplified test.

When applying between 100 and 80 ng of the rHBeAg in the macroporous cushion we obtained a good discrimination between the disappearance of the specific red sign of the positive plasmatic samples to the anti-HBeAg antibodies and the appearance of this sign in the samples that were negative to these antibodies in the immunochromatographic test without amplification. At between 60 ng and 40 ng of rHBeAg, the intensity of the red sign in the samples that were negative to anti-HBeAg antibodies was less than at 80 ng for the disappearance under 40 ng. In the rest of the experiments we used 80 ng of the rHBeAg in the macroporous cushion, and this represented the minimum amount of the recombinant antigen that will be neutralized by the antibodies anti-HBeAg in the plasma that were weakly positive in the non-amplified immunochromatographic test.

Optimization of the amplified immunochromatographic test

In the immunochromatographic assay for the detection of antibodies anti-HBeAg amplified with avidin-biotin we standardized the coating conditions of the avidin in the nitrocellulose strip and of the simultaneous application of the biotinylated CBHepB.AgE and the rHBeAg in the cushion of the conjugate. We achieved a better coating of 2 µg of the avidin in the nitrocellulose at basic pH compared to acid pH. This result agreed with a greater binding of the avidin to the nitrocellulose at a pH near its isoelectric point, which is about 10 to 10.6 [17].

In the use of the technology avidin-biotin a better relation biotinylated MAb should be used that will bind the avidin that is at the specific capture zone because an excess of the biotinylated antibody will not allow these antibodies to form a complex with the antigen and compete in the binding of the avidin with those that do form complexes with the antigen. If the amount of biotinylated antibodies without the antigen is larger than the amount of the biotinylated antibodies with the antigen, then we expect a red sign to appear that is hardly perceptible at a better relation between biotinylated Mab and avidin that should be found because an excess of the biotinylated antibodies would form complexes with the antigen and the biotinylated antibodies without antigen would compete with the antigen-biotinylated antibody complexes. The best relation found of CBHepB.AgE biotinylated and rHBeAg was of 100 to 400 ng of biotinylated MAb and between 40 to 80 ng of antigen (Figure 3). Therefore, in the other experiments we used the relation between 200 ng of the biotinylated MAb and 40 ng of rHBeAg. With this result we were able to neutralize 40 ng of HBeAg in the macroporous cushion by the human anti-HBeAg antibodies and finally detect plasmatic samples that are weakly positive to these antibodies, which increased the analytical sensitivity twice in elation to the non-amplified immunochromatographic test.

Comparison of the immunochromatographic tests prepared with the commercial immunochromatographic assay to detect anti-HBeAg antibodies

In the evaluation of the samples of plasma with an immunochromatographic test from Advanced Quality™ for the detection of antibodies anti-HBeAg we obtained 23 positive samples and 23 negative samples to these antibodies. The non-amplified immunochromatographic test presented a negative sample to the anti-HBeAg antibodies that was detected by the commercial system as positive. The kappa of Cohen statistics (kappa = 0.96) gave a value of 0.8 to 100, which is interpreted as an almost perfect agreement [18] between the non-amplified immunochromatographic test developed in the laboratory with the commercial test in the evaluation of these two groups of samples that were positive and negative to the presence of anti-HBeAg antibodies. That contradicting sample was analyzed in the non-amplified test with the addition of 0.25% to 10% (w/v) of the polymers PEG 8000 and Kucell as blocking agents for non-specificity and the red sign was not eliminated. The amplified immunochromatographic system was completely the same as the commercial one with the evaluation of these two groups of samples (Figure 4), which suggests a possible lack of sensitivity of the non-amplified immunochromatographic test in relation to the amplified test since in the former there should be more recombinant antigen neutralized (80 ng) than in the latter (40 ng) for the same amount of anti-HBeAg antibodies found in the sample. The other possible explanation is that there is no unspecific interaction favored in the amplified immunochromatographic test between the biotinylated CBHepB.AgE with the FAb-gold while this is so in the non-amplified immunochromatographic test of that conjugate with the non-

![Figure 4. Evaluation in an immunochromatographic test Advanced Quality to detect antibodies recognizing the antigens of the Hepatitis B virus (anti-HBeAg, 1 and 2) and in the immunochromatographic tests developed in the laboratory without (3 and 4) and with avidin-biotin amplification (5 and 6) of a negative (1, 3 and 5) and positive (2, 4 and 6) plasma samples for the anti-HBeAg antibodies. S is the well where the sample of the plasma is applied, T is the specific signal area and C is the control zone for the functioning of the test.](image)

biotinylated MAb. In this second test the use of 1 800 ng of non biotinylated CBHepB.AgE /2 µL represented 9 times more amount of the same antibody than in the first test where we applied 200 ng of biotinylated CBHepB.AgE /2 µL.

Interference of some biochemical compounds of the plasma in these immunochromatographic tests

The negative forms of the HBeAg and the HBCAg have a structural similarity of 149 aminoacids, but show antigenic differences [19]. That means that the antibodies anti-HBeAg generated in patients with HB do not recognize the native protein of HBcAg and the antibodies anti-HBeAg in a sick patient with this viral infection will not recognize the native protein of the HBcAg either. The HBcAg is a protein released in the bloodstream during the replication phase of the virus. However, the HBcAg of this virus is a very immunogenic protein that is retained mainly within the cell to form nucleocapsids of immature virions. In this study the selection process of the MAb anti-HBeAg was carried out with the recombiant proteins of the HBeAg and the HBcAg and as previously analyzed, there is crossed reactivity of the MAb CBHepB.AgE with the rHBcAg in solution and absorbed in the solid phase, for which reason it is important to study if there is interference of the plasmatic antibodies anti-HBeAg in the immunochromatographic tests carried out in the laboratory. Both immunochromatographic tests from the laboratory gave a red sign indicating the absence of anti-HBeAg antibodies in the 5 samples of plasma, that did not have antibodies anti-HBeAg and did have anti-HBeAg antibodies in the multiple commercial test. This result suggested the absence of interference between the human anti-HBeAg antibodies in these two immunochromatographic assays carried out in the laboratory. We also verified that the human plasmatic anti-HBeAg antibodies do not trap the HBcAg in spite of the structural aminoacidic similarity with the HBcAg and that these antibodies bind mainly to conformational epitopes produced by the spontaneous folding and assembly of several core proteins among themselves to form particles of 27 nm [2].

In the macroporous cushion of the amplified immunochromatographic test of the laboratory we applied the biotinylated CBHepB.AgE MAb together with the rHBeAg that will later be trapped in the nitrocellulose capture zone by the avidin. Biotin is a hydro-soluble vitamin that participates in the metabolism of the human body. This vitamin obtained from food is present in the blood of human beings and can interfere in the binding of the biotinylated antibody to the avidin absorbed in the nitrocellulose strip. Therefore, it was of interest to learn the concentrations of that vitamin that affect the functioning of this test. The amplified immunochromatographic test for the detection of anti-HBeAg antibodies did not produce a red signal in the specific capture zone when 100 ng/mL of biotin was added to the plasmatic sample negative to anti-HBeAg antibodies, in other words, that concentration of the vitamin inhibited the red signal that should appear in that test for that sample without anti-HBeAg antibodies. However, when to that negative sample to anti-HBeAg antibodies we added 20 ng/mL of biotin, the red signal did appear showing the absence of anti-HBeAg antibodies. In both cases the sample positive to antibodies anti-HBeAg did not give a red signal in the specific capture zone. This experiment showed that the amplified immunochromatographic trial does not have an interference of the biotin up until a concentration of 20 ng/mL in the plasma, which corresponds to a concentration that is 20 times greater than the therapeutic concentrations of this vitamin [20].

The interferon alpha 2b is a drug used in the treatment of chronic HB for which reason we should know if it can interfere in the determination of the antibodies anti-HBeAg. In these assays there was no interference of the recombinant interferon alpha 2b at 20 ng/mL and 100 ng/mL, both in the negative sample and in the positive sample for the anti-HBeAg antibodies. These concentrations that were added are 500 times greater that those detected in a randomized double blind pharmacokinetic study carried out with 24 healthy volunteers to which we injected a single dose of 10 x 106 IU of Intron A and Heberon alfa R (two formu-lations of recombinant human interferon alpha-2b) with the maximum serum activity values after 5 and 7 h for Intron A and Heberon alfa R, respectively [21].

Heparin is a sulfured mucopolysaccharide with the presence of sulphate and carboxyl groups in each disaccharide unit, which makes it strongly poly-anionic when in solution [22]. This molecule is synthesized in the body by the mastocytes, but it is also used as an anti-coagulant for the blood samples in the laboratory to obtain plasma. Since heparin is negatively charged it can interfere in the bonds of the gold particle (negatively charged) to the poly-L-lysine (positively charged) in the control zone of the functioning of the nitrocellulose of both immunochromatographic tests and the avidin (that may be positively charged) during the migration of the plasma at neutral pH in the specific capture zone of the amplified immunochromatographic test. The heparin at 2 IU/mL and 10 IU/mL in the plasma prevented the appearance of the red sign in the poly-L-lysine coated in the nitrocellulose when PAb-gold migrates through that zone in the two types of immunochromatographic tests, both for the samples that are positive and those that are negative to antibodies anti-HBeAg. The effect of the heparin in both concentrations used on the avidin (2 µg) did not produce the disappearance of the red sign in the no amplified immunochromatographic test and there is a good differentiation between the positive and negative samples to the anti-HBeAg antibodies. These concentrations of heparin correspond to those used in the laboratory as an anti-coagulant (2 IU/mL) and 5 times greater [13]. These 2 concentrations of heparin did not affect the disappearance of the red sign in the control zone for the functioning of the immunochromatographic tests when it was coated by the anti-immunoglobulin G from mice in sheep. In conclusion, the substitution of the poly-L-lysine by the anti-immunoglobulin G from mice in sheep in that zone of the control of the functioning makes it possible to use heparin as an anti-coagulant of the blood in both tests. This antibody anti-immunoglobulin G of mice has been
shown to be stable for 1 or 2 years in the accelerated stability experiments of an immunochromatographic test with a different specificity [15].

Accelerated thermostability of the different biological reagents used in the immunochromatographic tests carried out in the laboratory

The thermally accelerated stability studies predict the possible time interval in which the components of the immunochromatographic test in its different formulations may be stored at room temperature without affecting the quality of the test. This in no way substitutes the real time study of the stability of those components at between 20 and 25 °C; however, this may help give information to researchers to identify before time and with less resources the formulations tested that would ensure the preservation of the components of the immunochromatographic test. It has been said that a stability at 60 °C for 3 days of a component or of the complete immunochromatographic test is equivalent to a real time test of 8 to 12 months at room temperature and for 5 days it is equivalent of between 12 months and 18 months [23].

In the immunochromatographic test without amplification we studied the CBHepB.AgE and the PAb-gold. The CBHepB.AgE (1.8 µg) coated with the nitrocellulose was stable at 60 °C with trehalose 8% at 3 and 5 days. We used as the final diluent of the coating MAb Igepal 0.05% and trehalose 8% in the other experiments. The PAb-gold applied in the macroporous material with its diluent ½ was stable with trehalose 10% and 20% at 3 and 5 days (Figure 5). Therefore, trehalose 10% in the diluent of the conjugate was used in the other experiments.

In the amplified immunochromatographic test we studied the coating with avidin in the nitrocellulose and the simultaneous application of the biotinylated MAb and of the rHBeAg in the macroporous cushion. The avidin was stable with trehalose at 5 and 10% at 60 °C for 3 and 5 days, where the positive samples did not give a signal and the negative samples gave a red sign that is comparable with the strips stored at room temperature. The coating of the test with avidin at 0% of trehalose gave a red sign on evaluating a sample that is negative to antibodies anti-HBeAg, but less intense that the control at room temperature. Therefore, the formulation finally used for the avidin contained trehalose at 5%. The biotinylated CBHepB.AgE and the rHBeAg were stable with the trehalose 8% at 3 and 5 days. With 4% trehalose these components were not stable for 5 days, because a red sign was not obtained in the negative sample of anti-HBeAg antibodies. Given this result we used the concentration of 8% trehalose in the diluent of the biotinylated MAb and of the rHBeAg of the amplified immunochromatographic test and only of the rHBeAg in the non-amplified immunochromatographic test. The concentrations of the trehalose that guarantee the stability of the biological reagents used in these immunoassays ranged between 5 and 10%, which agree with those obtained in other immunochromatographic tests carried out in the laboratory with a different specificity to detect cardiac troponin I cardiaca in plasma [15] and were less than those reported in an immunochromatographic test for the detection of Salmonella Typhimurium where the concentration of trehalose of the formulation of the lyophilized biochemical compound in the macroporous material reached 24% [24].

In the patophysiology of HB there are three distinguishable phases: the immune tolerance phase, the immune clearance phase and the phase of the residual inactive carrier [1]. In the tolerance phase there is the surface antigen of the HB, the AgeHB, normal serum concentrations of hepatic transaminases and high levels of viral genetic load (>105 copies/mL). In the phase of immune clearance the viral genetic load remains high, but there is an increase in the serum concentrations of hepatic transaminases and the final point of this stage is indicated by the serum disappearance of the HBeAg and the appearance of antibodies anti-HBeAg. Later they pass to the final phase of the residual inactive carrier where the viral genetic load decreases to less than 104 copies/mL and the concentrations of hepatic transaminases decrease. It has been stated that the serum disappearance of the HBeAg and the appearance of the anti-HBeAg antibodies occurs spontaneously in between 50-70% of the patients with chronic HB and it is not so when mutants of HBV are selected. In this last case, chronic HB HBeAg-negative is produced in patients with the presence of serum anti-HBeAg antibodies that evolved with persistent or transitory increases of the viral genetic load and of the serum concentrations of the hepatic transaminases [25].

The importance of the appearance of antibodies anti-HBeAg together with the disappearance of the serum HBeAg is observed in marking an evolutionary phase in more than half the patients with chronic HB. And also those two immunological markers may be indicators of a favorable prognosis in patients that have responded to the antiviral and immunomodulatory treatment for which reason they have been used to evaluate the success of clinical trials with different combinations of antiviral agents in patients with chronic HB that are HBeAg positive [3, 25].

Conclusion

Knowing the information that can be contributed with the detection of antibodies anti-HBeAg in the studies of clinical trials of therapeutic agents for the treatment of HB, different methods have been used to detect antibodies anti-HBeAg in the blood plasma of patients with chronic HB. In this study we have used an immunochromatographic test for the detection of anti-HBeAg antibodies, which has been shown to be stable for 1 or 2 years in the accelerated stability experiments of an immunochromatographic test with a different specificity [15]. We have studied the thermally accelerated stability of the different biological reagents used in the immunochromatographic tests carried out in the laboratory. We have used a concentration of 8% trehalose in the diluent of the biotinylated MAb and of the rHBeAg of the amplified immunochromatographic test and only of the rHBeAg in the non-amplified immunochromatographic test. The concentrations of the trehalose that guarantee the stability of the biological reagents used in these immunoassays ranged between 5 and 10%, which agree with those obtained in other immunochromatographic tests carried out in the laboratory with a different specificity to detect cardiac troponin I cardiaca in plasma [15] and were less than those reported in an immunochromatographic test for the detection of Salmonella Typhimurium where the concentration of trehalose of the formulation of the lyophilized biochemical compound in the macroporous material reached 24% [24].

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treatment of patients with chronic HB that are HBeAg positive, we developed in the laboratory an immuno-
chromatographic system for the detection of anti-
HBeAg antibodies in the plasma. We studied two 
formats: one amplified with avidin-biotin and another 
one without amplification. The results obtained suggest 
the possibility of developing an immunochromatogra-
phic system under the laboratory conditions to detect 
antibodies anti-HBeAg in plasma with anti-coagulants 
of sodium citrate, heparin and EDTA. The avidin-
biotin used in the amplified immunochromatographic 
test improved the analytical sensitivity on applying 
a lower amount of AgeHBr to be neutralized than in 
the non-amplified one. The avidin may be used in the 
one step competitive immunochromatographic tests 
for the detection of anti-HBeAg antibodies in the 
plasma, in spite of the ionic charge and the presence 
of carbohydrates in their structure, without affecting 
the characteristics of the performance of these trials 
(specificity, sensitivity, speed, simplicity) and contri-
bute a more economic possibility than the application 
of the streptavidin with biotin.

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