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Impact Of Ligand Density On Hepatitis B Virus Surface Antigen Immunoaffinity Chromatography Efficiency And Ligand Leakage

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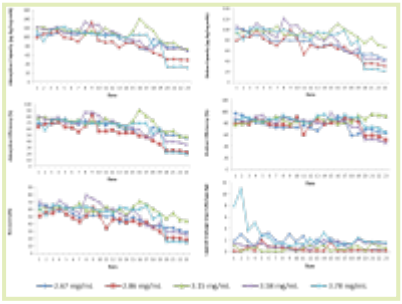

Stability in twenty-three purification cycles of the CB.Hep-1 mAb immunosorbents applying HBsAg purified samples

ABSTRACT

Hepatitis B surface antigen (HBsAg) can be purified by immunoaffinity chromatography for human vaccination, which involves key aspects such as support (Agarose) and ligand (monoclonal antibodies (mAb)). Theoretically, an increase in immunosorbent ligand density could provoke a decrease in elution capacity, elution efficiency and recovery of immunosorbents. To corroborate this hypothesis, the impact of five CB.Hep-1 mAb ligand densities (2.67 - 3.78 mg/mL) on HBsAg immunopurification capacity and ligand leakage was analyzed in 23 purification cycles applying purified HBsAg samples.

Next, ligand density that revealed better purification results applying purified HBsAg samples was challenged with HBsAg unpurified samples in five purification cycles. As results, 3.15 mg/mL-CB.Hep-1 mAb-immunosorbent showed 105.1 ± 16 µg HBsAg/mg mAb as adsorption capacity, 91.5 ± 10.4 µg HBsAg/mg mAb as elution capacity, 58.9 ± 6.6 % as HBsAg recovery, 69.5 ± 10.3 % as adsorption efficiency, 84.8 ± 6.8 % as elution efficiency and 0.57 ± 0.43 ng mAb/µg HBsAg as ligand leakage. The challenge of 3.15 mg/mL-CB.Hep-1 mAb-immunosorbent with HBsAg unpurified samples also evidenced significant differences ($p < 0.005$) in adsorption capacity, elution capacity, elution efficiency, recovery and mAb released from immunosorbent As conclusion, the ligand density increase has a significant negative impact on immunosorbent elution efficiency for HBsAg purification and 3.15 mg/mL is the most suitable ligand density for immunosorbent based on Zetarose CL4B to purify HBsAg allowing twenty-three purification cycles, a recovery higher than fifty percent, a ligand leakage lower than approved limit and a notable reduction in immunosorbent production process cost.

INTRODUCTION

According to the World Health Organization, there are more than 350 million Hepatitis B virus (HBV) chronic carriers

worldwide and approximately 25 % of the carriers can develop liver cirrhosis and carcinoma, being HBV responsible for the death of one million people annually (WHO, 2013).

The HBV has a spherical shape with a lipoprotein coating made up of different proteins, composed mostly of the Hepatitis B virus surface antigen (HBsAg) (Gish et al., 2015). According to this idea, the recombinant Hepatitis B vaccines use the HBsAg synthesized in yeasts or mammalian cells as active pharmaceutical ingredient. For such purpose, transformed cells are grown in industrial-scale fermentors where the HBsAg is expressed and assembled into immunogenic spherical particles that exposes the highly immunogenic “a” antigenic determinant (Gotthard et al., 2008).

The Center for Genetic Engineering and Biotechnology of Havana, Cuba is one of the worldwide leader institutions, where the HBsAg is produced to be used in the production of recombinant vaccines against Hepatitis B (Hardy et al., 2000). Due to this effort, Cuba is one of the few countries in the world that has the population below 15 years old vaccinated against the HBV and thus cases of Hepatitis B in children of this age has not been reported since 2007.

In the biomanufacturing process of the recombinant HBsAg, the main purification step is the immunoaffinity chromatography (Hardy et al., 2000). This affinity chromatography technique was firstly implemented in the 1930s and since the inception of the affinity chromatography about 50 years ago (Cuatrecasas, 1968), it has become an invaluable tool in life sciences (Subramanian, 2002; Fitzgerald et al., 2017), because it is possible to isolate proteins with a high purity degree in a single purification step facilitating the resolution of the subsequent purification steps and saving time and money (Moser and Hage, 2010).

The main components of the immunoaffinity chromatography are the support and the ligand, which are usually Agarose and monoclonal antibodies (mAb), respectively. The supports is selected on the basis of having a large surface area, controllable porosity, sufficiently hydrophilic character to avoid non-specific adsorption of contaminants and mechanical stability at high pressure (Wilchek and Miron,1999; Moser and Hage, 2010). On the other hand, the term ligand is usually used to refer to a molecule, which interacts with the target molecule. To be used as ligand, mAb are immobilized onto the Agarose, where a sample containing the target protein is then applied to be isolated (Linhardt et al., 1987; Fitzgerald et al., 2017). In this sense, the ligand density is crucial for the success of the immunoaffinity chromatography, because, authors of this study hypothesize, small changes in the ligand density could provoke multiple sites of protein adsorption, non-homogenous distribution of the ligand and mostly changes in the antigen adsorption capacity, antigen elution capacity and antigen recovery of the immunosorbents.

In this immunoaffinity chromatography, the Zetarose CL4B matrix is used in the immunosorbent production process, which, as Sepharose CL-4B, has a solid material content ranged 2 % - 4 %, and therefore the ligand density obtained at the end of the mAb coupling process, expressed as the amount of mAb per dried material weight can be very high (2 %) or high (4 %) or, causing an undesired lot to lot variation of the immunosorbent purification capacity and ligand leakage.

In addition, the control of the real ligand density has to be even more accurate; when the affinity constant of the mAb used as ligand is high (> 108 M-1) and the antigenic epitope recognized by the mAb paratope is repeated in the same antigen molecule or particle. In this case, the total strength of the interaction that considers all epitopes and paratopes involved in the interaction is called avidity, which is much greater (about 1 - 2 orders) than the affinity constant value “per se”; because different interactions play a role to stabilized the interaction between the antigen and antibody (Landry et al., 2015).

The CB.Hep-1 mAb is a mouse gamma-immunoglobulin (IgG2k) specific for the “a” antigenic determinant of the HBsAg. The specific sequence recognized by the paratope of this mAb was clarified years ago and it is located in the first loop of the “a” determinant (Fernández de Cossio et al., 1997). This mAb has a high affinity constant ranged 109 - 1010 M-1 (Valdés et al., 2009). On the other hand, the HBsAg originally described as the Australia antigen in sera of patients infected with Hepatitis B virus shows a spherical shape and 21 nm in diameter, or a filamentous and tubular shape, approximately 21 nm across and up to several hundred nm in length. The construction in yeasts of the recombinant HBsAg has shown similar properties to HBsAg isolated from human sera. An electron microscopy view shows a complex macromolecular aggregate composed of proteins (75 % weigh) and carbohydrates (25 % weigh). The HBsAg amino acid sequence and composition is responsible for the induction of antibodies, where the “a” antigenic determinant is the main epitope described to rise a protective immune humoral response (Golsaz-Shirazi et al., 2016; Joan et al., 2020). It has been reported that the 22 nm HBsAg particles are assembled from about 100-120 monomers (Ganem and Varmus, 1987), which indicate the epitope recognized by the paratope of the CB.Hep-1 mAb can be repeated up to 100 - 120 times in each HBsAg particle.

Therefore, the subject of this paper was to study the impact of the CB.Hep-1 ligand density on the efficiency and the ligand

leakage of the immunoaffinity chromatography performed to purify the HBsAg produced in Pichia pastoris for human use.

MATERIALS AND METHODS

Production of ascites rich in the CB.Hep-1 mAb

The murine hybridoma 48/1/5/4, secretor of the CB.Hep-1 mAb was generated by the fusion of spleen cells of BALB/c mice, immunized with a natural preparation of the HBsAg, with myeloma cells Sp2/0-Ag14. For ascites production, one million of cells were intraperitoneally inoculated in 22-24 g Balb/c mice (Fontirrochi et al., 1993).

Purification of the CB.Hep-1 mAb

The ascites stored at -20 °C was thawed at 37 °C, clarified and filtrated by 0.45 - 0.22 µm capsule before to be applied to an affinity chromatographic column. The affinity chromatography was performed on a BPG 100/500 column (Amersham-Biosciences, Uppsala, Sweden) packed with Protein A Sepharose Fast Flow (General Electric Healthcare, Uppsala, Sweden). The equilibrium and washing buffer were done with 150 mmol/L phosphate buffered saline (PBS), pH 8.0 at 100 cm/h as linear flow and the CB.Hep-1 mAb was eluted from the column with 0.1 mol/L citric acid, pH 5.0 and 0.1 mol/L citric acid, pH 3.0 at a linear flow of 100 cm/h, respectively. Next, the elution sample were incubated with the second elution buffer for 1 h and neutralized with 2 mol/L Tris (Merck, Danmstadt, Germany) under gentle agitation. Subsequently, a sample buffer exchange was done to 0.020 mol/L Tris/0.150 mol/L NaCl, pH 7.6 in a BPG 200/750 column packed with 21 L of Sephadex G-25 (Amersham-Pharmacia, Uppsala, Sweden). Later, the material obtained was concentrated by ultrafiltration (Sartorius, Göttingen, Germany) and filtered by 0.22 ?m filtration capsule under sterile conditions.

Zetarose CL4B activation with cyanogen bromide

Zetarose CL4B (Emp-BIOTECH, Buch Germany) activation with BrCN was performed following the modify activation procedure reported by Axen, Porath and Ernback (Porath et al., 1967). As modification, the drying step with 40 % acetone was eliminated.

Immobilization of CB.Hep-1 mAb to BrCN-Zetarose CL4B

The buffer of the purified samples of the CB.Hep-1 mAb was firstly exchanged to 100 mmol/L Na2CO3/ NaHCO3/500 mmol/L NaCl; pH 8.3 by gel filtration chromatography in a column packed with 21 L of Sephadex G-25 coarse (Amersham-Pharmacia, Uppsala, Sweden). Then, the activated matrix was hydrated with 1 mmol/L HCl in a ratio of 5 L/L of matrix and equilibrated with 100 mmol/L Na2CO3/NaHCO3/500 mmol/L NaCl, pH 8.3 in a 1.5 L/L of matrix ratio. Next, the mAb solution was added to obtain the desired ligand density (2.80, 3.00, 3.20, 3.60, 3.80 mg/mL) and stirred for 2 h at 23 °C. Free active groups were neutralized with 200 mmol/L glycine; pH 8.0 in a ratio of 2 L/L of matrix and finally, two alternate washes were performed with sodium acetate 100 mmol/L/500 mmol/L NaCl, pH 4.0 and 100 mmol/L Na2CO3/NaHCO3/500 mmol/L NaCl, pH 8.3 (1 L/L of matrix) and the respective immunosorbents were store in 150 mmol/L PBS, pH 7.2/0.01 % Thimerosal.

Immunoaffinity chromatography performed to evaluate CB.Hep-1 mAb immunosorbent performance with purified HBsAg samples

The immunoaffinity chromatography was performed in PD-10 columns packed with 8.14 mL of immunosorbent, equilibrated with 45 mL of 20 mmol/L Tris/3 mmol/L EDTA/0.5 mol/L NaCl, pH 7.39 at a linear flow rate of 4.65 cm/h. Samples were applied at a theoretical ratio of 155 ?g HBsAg/mg mAb and 4.65 cm/h as linear flow rate, the applied amount was 3.50 mL; 3.80 mL; 4.10 mL; 4.70 mL and 6.20 mL for each ligand density (2.80, 3.00, 3.20, 3.60, 3.80 mg/mL) respectively. The washing step was carried out with 20 mmol/L Tris/3 mmol/L EDTA/1 mol/L NaCl; pH 7.38 at 7.67 cm/h of linear flow rate. The elution was carried out using 20 mmol/L Tris/3 mmol/L EDTA/0.5 mol/L NaCl/3 mo/L KSCN, pH at 7.76 cm/h of linear flow of 7.67 cm/h as an eluting agent. This study was done in 23 purification cycles.

Challenge of the 3.20 mg/mL-CB.Hep-1 mAb immunosorbent with unpurified HBsAg samples

Unpurified HBsAg samples were applied directly to the immunosorbent packed in PD-10 columns at a ratio of 155 ?g HBsAg/mg mAb and 4.65 cm/h as linear flow rate, the applied amount was 5.06 mL. The washing step was carried out with 20 mmol/L Tris/3 mmol/L EDTA/1 mol/L NaCl, pH 7.38 at 7.67 cm/h of linear flow rate. The elution was carried out using 20

mmol/L Tris/3 mmol/L EDTA/0.5 mol/L NaCl/3 mol/L KSCN, pH at 7.76 cm/h of linear flow of 7.67 cm/h as an eluting agent. The regeneration of the matrices was performed after each purification cycle by applying 60 mL of 0.1 mol/L Tris/0.5 mol/L NaCl, pH 8.51, 100 mL of purified water, 60 mL of 0.1 mol/L sodium acetate/0.5 mol/L NaCl, pH 4.52, 100 mL purified water. The behavior of the adsorption capacity, adsorption efficiency, elution capacity, elution efficiency, recovery, ligand leakage and purity were measured in 5 purification cycles.

DETERMINATION OF CHROMATOGRAPHIC PARAMETERS

The mathematical expressions used to determinate key chromatographic parameters are described below:

$$\text{Ligand density} = \frac{(\text{LD expected} \cdot \text{gel volume}) - \text{not adsorpted mass}}{\text{gel volume}}$$

$$\text{mAb coupled amount} = \text{LD} \cdot \text{gel volume}$$

$$\text{Adsorption capacity} = \frac{\text{adsorpted mass}}{\text{coupled mAb mass}}$$

$$\text{Elution capacity} = \frac{\text{eluted mass}}{\text{coupled mAb mass}}$$

$$\text{Adsorption efficieincy} = \frac{\text{adsorpted mass}}{\text{initial mass}} \text{ x } 100$$

$$\text{Eulition effciciency} = \frac{\text{eluted mass}}{\text{adsorpted mass}} \text{ x } 100$$

$$\text{Recovery} = \frac{\text{eluted mass}}{\text{initial mass}} \quad \text{x } 100$$

Benefit-Cost analysis

The benefit-cost ratio (BCR) was calculated using the next expression

$$\text{BCR} = \frac{(\text{Income 2} - \text{Income 1})}{(\text{Operation costs 2} + \text{Investment costs 2} - \text{Operation costs 1})}$$

Quantification of the CB.Hep-1 mAb by Enzyme-linked immunosorbent assay (ELISA)

Polystyrene plates were coated for 20 min at 50 ± 2 °C with 10 ?g/mL of HBsAg (100 ?L/well) in 0.100 mol/L Na2CO3/NaHCO3, pH 9.6. Next, 100 ?L of samples were applied to each well including the standard curve points and controls. The plates were placed in a humid chamber and incubated 1 h at 37 ± 2 °C. At the end of this time, plates were washed 4 times with washing solution (0.150 mol/L PBS/0.1 % Tween-20) and incubated for 1 h at 37 ± 2 °C with a polyclonal anti-mouse IgG sheep antibody preparation conjugated with horseradish peroxidase (SIGMA, St. Louis, Missouri, USA). Then, plates were washed again 5 times with washing solution and the reaction was developed with 100 ?L of substrate solution (5 mg of Orthophenylenediamine (OPD), 5 ?L of hydrogen peroxide, 10 mL of substrate buffer (0.090 mol/L C6H8O7.H2O, 0.200 mol/L Na2HPO4; pH 5.5) (SIGMA, St. Louis, Missouri, USA). The reaction was stopped at 20 min with 50 ?L of 1.5 mol/L H2SO4 and the absorbance was determined at 492 nm in a microELISA reader (TiTertek, Multiskan MC340) (Valdés et al., 2009).

Total protein quantification

The total protein concentration was determined by Lowry method using bovine serum albumin as reference material (Lowry, 1951). The range of the standard curve used was 100 to 500 g/mL.

Determination of HBsAg concentration by optical density

The HBsAg absorbance was measured at 280 nm on an UV-Visible Spectrophotometer (Pharmacia Biotech Ultrospec 2000, Cambridge, England). The antigen concentration was done by mean of the expression:

$$C_{HBsAg} = \frac{Abs}{5}$$

Where:

C_{HBsAg}: concentration of HBsAg (mg/mL)

Abs: absorbance of the sample

5: molar extinction coefficient

Determination of HBsAg purity by SDS-PAGE

The purity of the HBsAg was determined following the procedure described by Laemli (1970).

Determination of ligand leakage

Plates (Costar, Cambridge, Mass., USA) were covered with sheep polyclonal anti-mouse antibodies overnight at 2 - 8 °C. Then, plates were blocked for 30 min at 37 ± 2 °C. Plates was washed and samples eluted from immunosorbents were added and incubated for 3 h at 37 ± 2 °C with 1 % skimmed milk/150 mmol/L PBS, pH 8.0. After the washing step, plates were incubated with 100 ?L per well of a goat anti-mouse polyclonal antibody-horseradish conjugated peroxidase (SIGMA, St. Louis, Missouri, USA). The reaction was developed using 100 ?L/well of 0.05 % OPD and 0.015 % H2O2 in citrate buffer, pH 5.0 and stopped with 50 ?L of 1.25 mol/L H2SO4 well. The absorbance was measured in a Multiskan ELISA reader using a 492 nm filter (Valdés et al., 2009).

Determination of carbohydrate content in immunoaffinity chromatography eluates

Carbohydrates were determined according to method described by (Carney, 1986). The spectrophotometric determination of the total carbohydrate content was based on the hydrolysis and acid degradation of the sugars with the formation of the furfural, 5 hydroxymethylfurfural and derivatives in its reaction with the anthrone in a strongly acid medium.

Determination of lipids in immunoaffinity chromatography eluates

Lipids determination was based on the lipid reaction with sulfuric acid/phosphoric acid and vanillin following the procedure described by (Woodman and Price, 1972).

Statistical and variable analysis

For each experimental run, the value of all determinations was averaged, and the statistical processing of the data was carried out by means of a Hypothesis Test, ANOVA and Kruskal-Wallis test. The level of significance applied was always 95 %. In all cases, the programs used were Statgraphic Centurion XV Version 15.2.06 (Statistical Graphics Corp., USA) and Microsoft Excel.

RESULTS AND DISCUSSION

Immobilization of biomolecules on solid surfaces has been widely used for different applications. Among these applications are affinity chromatography and analytical techniques (Hage, 1998; Hage, 1999). Affinity chromatography was introduced almost

50 years ago and, so far, is the most powerful tool to purify biologically active molecules (Cuatrecasas, 1968; Hage et al., 2012). This chromatography technique revolutionized the molecular biology, biochemistry, medicine and biotechnology. In general terms, the affinity chromatography is a method created for separating biochemical mixtures based on a highly specific interaction between antigen-antibody, enzyme-substrate, or receptor-ligand. In specific terms, the high selectivity of affinity chromatography is caused by allowing the interaction of the target molecule with the stationary phase in order to be separated from the undesired material, which will not elute first. Then, the target protein will be eluted from the solid support under the presence of the eluting solutions or solvents (Firer, 2001).

Among the most popular affinity-derived technologies is the immunoaffinity chromatography on antibody columns to purify antigens. One of the reasons for the rapid expansion of immunoaffinity chromatography was advances in the generation of monoclonal antibodies (Köhler and Milstein, 1975). This technology makes possible the disposal of an unlimited number of ligands, since mAb can be produced against almost any compound. Currently, it seems that the immunoaffinity chromatography application will continue to grow; at least for laboratory technologies to isolate target proteins with high purity in one single step helping to proof the concept of the target protein in terms of biological activity, before looking for a large-scale purification method of the target protein. However, the high cost of the mAb production and ligand leakage, within other factors could limit the large-scale use of this chromatography, at least for the purification of proteins for pharmaceutical uses.

A comparative large number of support materials for affinity chromatography are commercially available Gustavsson and Larsson, 2006). Within them, the most popular support used is the Agarose. The Agarose is a useful material for chromatography, because it does not absorb biomolecules to a significant extent, has good flow properties, and can tolerate extremes of pH, ionic strength and denaturants. Within the examples of Agarose-based matrices are Sepharose and WorkBeads 40 SEC (cross-linked beaded Agarose), Praesto and Superose (highly cross-linked beaded Agaroses), and Superdex (dextran covalently linked to Agarose).

On the other hand, there are several methods for ligand immobilization (Hermanson, 2013). Immobilization of proteins on Agarose is complex due in part to the combination of interacting forces. The principle was originally demonstrated by a highly reactive cyanate ester (Kohn and Wilchek, 1982a; Kohn and Wilchek, 1982b). In this support, the BrCN activation method is the most successfully method used for the attachment of ligands that bind later the target proteins. The ligands are linked covalently to activated hydroxyl groups of Agarose beads and the target protein to the ligand by means of the isourea bound.

Conversely, the charge of the isourea bound may cause instability of the isourea linkage with the ligand and thus several problems during the purification of the target proteins could be produced by a constant leakage of the bound antibody provoking the contamination of the target protein with undesired ligand traces. Therefore, the multipoint attachment of the antibody is needed, to which the higher amount of the active groups combined with the smallest ligand density can contribute to reduce the ligand leakage.

Analogously, the characteristic of the antigen-antibody complex involved in the immunoaffinity chromatography is also crucial. The antibody affinity constant is a critical parameter for this kind of chromatography, since it implies using harsh antigen elution conditions, which may increase the ligand leakage, loss of antigen recognition capacity of the ligand and most likely damages in the support structure as well. This is even more critical if antibodies with high affinity and multiepitopic antigens are involved in the immunoaffinity chromatography. In these cases, avidity is perhaps the most important parameter, because it increases the strength of the antibody-antigen interaction over the affinity constant value.

Unfortunately; this state is the condition that characterizes the interaction between the CB.Hep-1 mAb and the HBsAg (Valdés et al., 2009). That is why, in this report, the impact of the CB.Hep-1 mAb ligand density on the HBsAg immunoaffinity chromatography efficiency and ligand leakage was studied with the aim to increase the elution capacity and efficiency.

For such goal, a modified Zetarose CL4B activation procedure with BrCN was firstly introduced to keep Zetarose CL4B hydrated during the whole BrCN activation procedure. Modifications were done, because an optimized protocol for Zetarose CL4B has not been validated yet. The BrCN activation protocol used in the study was previously standardized for Sepharose CL-4B. But, slight differences in the Zetarose CL4B behavior have been observed when this matrix is activated using exactly the same BrCN activation procedure optimized and applied during 30 years at industrial scale for BrCN-Sepharose CL-4B by the authors's team.

Concerning to results, mAb coupling efficiency and true-expected ligand density ratio for Zetarose CL4B ranged 90.81 – 95.29 % and 95.36 – 99.44 %, respectively in this study. These results are not coincident with values reported for the Sepharose CL4B BrCN activation method (Hernández et al., 2001). As no correlation between the expected ligand density and coupling

efficiency was estimated (R2= 0.1292), authors postulate that these relative low coupling efficiency could likely be explained by the fact that the mAb concentration in the coupling reaction was always below the expected ligand density (Table 1). According to previous experiences, higher values of mAb coupling efficiency have been obtained when the mAb concentration in the coupling reaction is equal or higher than the expected ligand density. Another explanation could be that the number of active groups was insufficient. However, this cannot be applied since the BrCN activated support was the same for all studied immunosorbents, which would definitively provoke the same ligand density for all immunosorbents. Therefore, this second explanation could be discarded.

Summarizing, the coupling efficiency reached allowed obtaining immunosorbents with 2.68, 2.86, 3.15, 3.58 and 3.78 mg/mL, which represent a number of mAb molecules per bead of support equivalent to 6.36x1010; 6.81x1010; 7.31x1010; 8.58x1010 and 1.13x1011, respectively (Table 1). This amount of mAb molecule per bead of support (nearly 63 - 113 billions of mAb molecules per Agarose bead) corresponded with those calculated by (Hayworth and Hermanson). As consequence, a maximum difference of 1.78 fold between the ligand density 3.78 mg/mL and 2.68 mg/mL was calculated.

In regards to the immunoaffinity chromatography efficiency, Figure 1 shows a graphic representation of 23 purification cycle profiles applying previously purified HBsAg samples in the studied immunosorbents at an average ratio of application equal to 138.2±13.9 µg HBsAg/mg mAb). The antigen application rate is important because higher values (>250 µg HBsAg/mg mAb) can provoke a decreases in the antigen elution capacity and efficiency. The profiles of the adsorption capacity, elution capacity, adsorption efficiency, elution efficiency and recovery showed the same trend, characterized a progressive decrease of the values purification cycle, demonstrating that this support presents limitations to this immunoaffinity chromatography and thus a relative short stability to purity the HBsAg particles. An immunoaffinity chromatography should show a higher number of purification cycles to be cost-effective. For instance Protein A chromatography is stable for more than 100 purification cycles. However, these values and trends do not different to those obtained with Sepharose CL4B and 4.0 mg/mL in general (< 19 purification cycles). In this case, the worst ligand density in terms of adsorption capacity was 3.15 mg/mL, which showed a drastic decrease of the adsorption capacity at purification cycle 8 (less than 10% of value of the first purification cycle). On the contrary, the most stable were 2.68 mg/mL and 3.78 mg/mL (Table 2).

As it can be also note in Figure 1 and Table 2, all immunosorbents showed a relative similar trend respect to the elution capacity of the HBsAg, which allow concluding that the efficiency of the immunosorbents was more dependent on the support characteristics than the ligand densities. Difference in the slope of the curves was only detected in the elution capacity of the 3.15 mg/mL-CB.Hep-1 mAb-immunosorbent (y= - 0.871x + 103.1). Equations that describes the behavior of the rest of the immunosorbent was (2.67 mg/mL-CB.Hep-1 mAb-immunosorbent, y= -3.343x + 101.7, 2.86 mg/mL-CB.Hep-1 mAb-immunosorbent, y= -3.536x + 97.59, 3.58 mg/mL-CB.Hep-1 mAb-immunosorbent, y= -3.128x + 119.96 and 3.78 mg/mL-CB.Hep-1 mAb-immunosorbent, y= -3.019x + 97.59. The most stable immunosorbent was the 3.15 mg/mL-CB.Hep-1 mAb immunosorbent, which showed a stable reduction in the elution capacity at the purification cycle 20. The less stable were immunosorbents of the ligand densities 2.67 mg/mL (purification cycle 4), 2.86 mg/mL, (purification cycle 11) and 3.58 mg/mL, (purification cycle 11).

The ratio pore size of the support and HBsAg size is against the relative free diffusion of the HBsAg into the bead, which is even more affected by the amount of mAb coupled to the matrix (Table 1). It has been reported that the pore size of the support must be at least 20 times greater than the size of the target protein to achieve a good diffusion of the target protein into the support pore. In the case of Zetarose CL4B and HBsAg; this ratio is almost 10 times, since the pore size expressed as fractionation rage of the matrix for globular proteins is about 20,000,000 Dalton and of the HBsAg is 2, 000, 0000 million Dalton. Another factor that manages the amount of HBsAg able to get into and out of the pores is the chromatography condition (flow rate and target protein concentration in the mobile phase). For instance, the strong elution condition used (Caotropic agent, 3 M KSCN) could irreversible damage the immobilized antibody purification cycle by cycle, because it disrupt the stability of the water interfering with hydrophobic interactions and thus the adsorption capacity and elution capacity.

Concerning mAb leakage; most random immobilization procedure, like BrCN-Zetarose CL4B, occurs through exposed residues such as lysine, which are in general located on the surface and not in the paratope of the mAb. This chemical condition provokes a multipoint attachment of mAb and as consequence a reduction in the antigen recognition capacity by conformational changes that compromise the paratope of mAb, and also a drastic reduction in the ligand leakage. Figure 1 illustrates the profile of the mAb leakage, when HBsAg non-purified samples were applied. The percentage of the mAb released from the column respect to the amount of mAb coupled ranged 0.02 % - 0.06 % [0.06 ± 0.04 % (LD= 2.67 mg/mL), 0.02 ± 0.02 % (LD= 2.86 mg/mL), 0.02 ± 0.01 % (LD= 3.15 mg/mL); 0.04 ± 0.01 % (LD= 3.58 mg/mL, 0.06 ± 0.05 % (LD= 3.78 mg/mL)]. It means that the amount of mAb retained in the support per each purification cycle was higher than 99 % in all

cases. As a consequence, the majority of the values were below the approved limit (3 ng mAb/µg HBsAg). The lowest values were observed in 2.86 mg/mL- and 3.15 mg/mL-CB.Hep-1 mAb, where all values were below the approved limit in the 23 purification cycles. On the contrary, the highest values were detected in the minimum and highest ligand density (2.67 and 3.78 mg/mL). In the case of the 2.67 mg/mL CB.Hep-1 mAb-immunosorbent, the higher values of mAb leakage were detected as result of a relatively low antigen elution, and thus the ratio mAb/HBsAg was higher. In the highest ligand density, the ligand leakage was higher most likely due to the existence of less multipoint attachment sites in regard to the higher number (1.78 fold) of molecule (Table 1).

To continue this study and considering preliminary experiment, results of the 3.15 mg/L-CB.Hep-1 mAb-immunosorbent applying purified samples of HBsAg were compared to those applying unpurified samples of HBsAg. For this last study, immunosorbents with the smallest ligand density 2.67 mg/mL and 2.86 mg/L were not selected, because the elution amount of HBsAg was very low in regard to other immunosorbents (1.66 ± 0.37 mg and 1.65 ± 0.46 mg, respectively) (Table 3). The challenge with different sample characteristics (purified and unpurified) was done, because researchers involved of the study have observed in previous experiences differences in the behavior of the immunosorbents when those are challenged with different application samples of HBsAg. For instance, if the HBsAg particles are aggregated in the application conditions, then the diffusion of the HBsAg particles into the bead will be even more affected and thus the chromatography efficiency as well. The characteristic of the unpurified samples of HBsAg are described in (Hardy et al., 2000).

As a result, statistical differences were detected in the HBsAg adsorbed amount [(purified sample, 3.09 ± 0.07 mg), (unpurified sample, 3.90 ± 0.24 mg), p=0.0090], HBsAg eluted amount [(purified sample, 2.51 ± 0.07 mg), (unpurified sample, 4.18 ± 0.43 mg), p=0.0090], average adsorption capacity [(purified sample, 118.50 ± 2.74 µg HBsAg/mg mAb), (unpurified sample, 149.61 ± 0.01 µg HBsAg/mg mAb), p=0.0090], average elution capacity [unpurified sample, 96.30 ± 2.67 µg HBsAg/mg mAb), (unpurified sample, 160.31 ± 0.02 µg HBsAg/mg mAb), p=0.0090], elution efficiency [(purified sample, 78.50 ± 8.89 %), (unpurified sample, 104.29 ± 8.63 %), p=0.0090], HBsAg recovery [(purified sample, 62.00 ± 1.54 %), (unpurified sample, 82.53 ± 8.59 %), p=0.0090] and mAb amount released from the column [(purified sample, 1303.00 ± 1168.52 ng), (unpurified sample, 4357.35 ± 3460.52 ng), p=0.00462]. Therefore, the result of these parameters was quite dependent on the characteristic of the application sample. In the case of the amount of mAb released from the column, differences could be explained by degradation of mAb coupled to the matrix theoretically produced by the action of proteases that could be present in the applying material. Nevertheless, data of mAb released from the column were characterized by a high dispersion of values; therefore, the number of determination should be increased to reach a clear conclusion on this topic.

On the contrary, the adsorption efficiency and ligand leakage expressed per amount of eluted HBsAg were not statistically affected by the applied material characteristics [(purified sample, 76.50 ± 1.71 %), (unpurified sample, 77.00 ± 4.85 %), p=0.08000]; [(purified sample, 0.57 ± 0.43 µg HBsAg/mg mAb), (unpurified sample, 1.00 ± 0.80 %), p=0.2660], respectively. Result of the mAb leakage was below of the approved limit (3 µg HBsAg/mg mAb) and the lack of statistical differences with results obtained applying purified sample experiment was produced by a higher HBsAg elution from column.

The elution improvement observed with the application of unpurified samples could be likely explained by differences in the density of the applied samples and by the influence of the contaminants on the diffusion of the HBsAg into the beads. Perhaps, under these conditions the antigen interacts more with mAb located at the surface of the bead or around the support pores. Therefore, less interference in the release of the HBsAg from the column could be produced. This increase in the elution efficiency brought as consequence an important increase in the HBsAg recovery 20.53 % (Table 4), which could allow producing 20 % more of vaccine doses.

The other important parameters measured in the immunosorbent challenged with unpurified samples of HBsAg were the purity of the eluted antigen, carbohydrate content and lipid content. In regard to the purity of the eluted antigen, this parameter is critical, because the subsequent purification steps were selected to increase the HBsAg purity level to values approved for an active pharmaceutical ingredient. In such sense, to reach the approved purity value of the active pharmaceutical ingredient, the purity of the HBsAg eluted from the immunoaffinity chromatography column should be higher ? 80 % (Hardy et al., 2000).

Concerning to this, a typical affinity chromatography chromatogram was observed in all purification cycles in this study. The chromatograms were characterized by two peaks. The first wide peak corresponded with the non-retained sample components that pass through the column during the application of the sample and washing of the column, while the second narrowed peak corresponded with HBsAg eluted by the action of the chaotropic agent 3 M KSCN. Hence, the purity of the HBsAg was higher than 90 % (average value = 92.6 ± 1.67 %) with a sample profile similar to the HBsAg reference material profile, characterized by a monomer (24 kDa) and a dimer band (48 kDa) (Table 4). These results are coincident with those

reported by (Hardy et al., 2000; Valdés et al., 2010). The other 8 % of the bands (do not include in monomer and dimer band) seems to correspond with the trimer of the HBsAg, but it cannot be concluded, since a western-blot with HBsAg specific antibodies was not performed to confirm the nature of these bands. Therefore, the reduction of the ligand density until 3.15 mg/mL did not affect the purity of the eluted antigen from the column (Figure 2) and guarantee the purity of the active pharmaceutical ingredient.

On the other hand, the content of carbohydrates and lipids was also measured in the 3.15 mg/mL-CB.Hep-1 mAb-immunosorbent eluates. The removal capacity of carbohydrates and lipids has to be studied, because Hepatitis B vaccines should be free or has a very low level of these two contaminants. Results of the carbohydrate measurements [average carbohydrate content= 6.40 ± 2.07 ng/µg HBsAg (Table 4)] revealed an extraordinary capacity of Zetarose CL-4B to remove carbohydrates, showing removal values higher than those obtained when Sepharose CL-4B has been used as support (Hardy et al., 2000). This average value is equivalent to (average carbohydrate content per vaccine dose= 0.128 ± 0.04 µg/20 µg HBsAg). This carbohydrate content was 23 fold lower than the vaccine approval limit (3 µg/20 µg HBsAg).

The average lipid content was 0.67 ± 0.37µg/µg HBsAg), which corresponded with a lipid content per vaccine dose of 13.38 ± 7.48 µg/20 µg HBsAg).These results are similar to those (14.0 ±0.28 µg/20 µg HBsAg) reported by (Hardy et al., 2000) using Sepharose CL-4B as support and is very close to the vaccine approval limit (25 µg/20 µg HBsAg). Therefore, as designer of the HBsAg downstream process have issued, the subsequent purification steps are unnecessary to remove carbohydrates and lipids and thus, as with Sepharose CL4B, immunosorbents based on Zetarose CL4B allow to fulfill with these two important quality specifications for human vaccination as well.

Finally, the BCR is a technique used to obtain the highest and best results at the least effort made. This effort includes: the investment of economic or physical resources, technical efficiency and human motivation. In this study, calculations were done for the ligand densities 3.78 mg/mL and 3.15 mg/mL taking one year and 0.75 USD as estimated value of the vaccine dose as the basis for calculation. The comparison of the main indicators for both processes is shown in Table 5. The BCR revealed a value greater than 1, which implies that the income is greater than the expenses, so the project done to reduce the ligand density was feasible. This analysis evidenced 16.7 of reduction in the cost of the 3.15 mg/mL-CB.Hep-1 mAb-immunosorbent in comparison with 3.78 mg/mL-CB.Hep-1-mAb-immunosorbent, which is considered a high reduction if the very high costs of mAb production (ligand) is taken into account.

CONCLUSIONS

The application of purified samples of HBsAg allowed concluding that the increase in the ligand density has a significant positive impact on the HBsAg adsorbed amount (2.18 - 3.57 mg). The 3.15 mg/mL- CB.Hep-1-mAb-immunosorbent shows the highest adsorption capacity, adsorption efficiency, elution capacity, and antigen recovery. The 3.15 mg/mL-CB.Hep-1-mAb-immunosorbent and the 2.86 mg/mL-Cb.Hep-1-mAb-immunosorbent show the lowest values of mAb released from the columns and ligand leakage. The 3.78 mg/mL-mAb-immunosorbent shows the highest HBsAg adsorbed and eluted amount and mAb released from the columns and ligand leakage. The ligand leakage is directly proportional to the ligand density of the immunosorbents from 2.86 to 3.78 mg/mL of ligand density. The most stable immunosorbent is the 3.25 mg/mL CB.Hep-1 mAb immunosorbent.

The application of unpurified samples of HBsAg to the immunosorbent has a significant impact on the HBsAg adsorbed amount, HBsAg eluted amount, adsorption capacity and elution capacity, elution efficiency, HBsAg recovery and mAb released from the column; but it does not has significant impact on adsorption efficiency, and ligand leakage per amount of eluted antigen (likely due to the dispersion of values). The 3.15 mg/mL CB.Hep-1-mAb-immunosorbent shows a high removal factor for carbohydrates and lipids. The ligand density 3.15 mg/mL of mAb is the most suitable ligand density for immunosorbent based on Zetarose CL4B to purify HBsAg allowing twenty-three purification cycles with an average recovery higher than fifty percent, a ligand leakage lower than the approved limit (3 ng mAb/µg HBsAg) and a notable reduction in the immunosorbent production process cost in comparison with higher ligand densities.

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CONFLICT OF INTERESTS

Authors declare that there is not conflict of interest to publishing the information in this journal and this information has not been published anywhere.

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Expected Ligand Density (mg/mL)	Volume of Zetarose CL4B in Coupling Reaction (mL)	mAb Concentration in Coupling Reaction (mg/mL)	mAb Coupling Efficiency (%)	True Ligand Density (mg/mL)	True-Expected Ligand Density Ratio (%)	Number of mAb Molecule per Bead of Support (# mAb/b)	Fold to respect the smallest ligand density (fold)
2.80	10	1.97	90.81	2.67	95.36	$6.36x10^{10}$	-
3.00	10	2.07	94.64	2.86	95.33	$6.81x10^{10}$	1.07
3.20	10	2.17	95.29	3.15	98.43	$7.31x10^{10}$	1.15
3.60	10	2.34	93.83	3.58	99.44	$8.58x10^{10}$	1.35
3.80	10	2.42	93.64	3.78	99.47	$1.13x10^{11}$	1.78

Table 1. Results of the CB.Hep-1 mAb coupling efficiency in Zetarose CL4B

Runs and Ligand Density (mg/mL)	Adsorption Capacity (%)					Elution Capacity (%)	
	2.67	**2.86**	**3.15**	**3.58**	**3.78**	**2.67**	**2.86**
1	100	100	100	100	100	100	100
2	2.51	7.47	1.34	-2.7	-20	-0.43	9.9
3	0.84	10.79	-3.66	-3.24	2.22	-9.96	9.38
4	3.35	13.28	-2.1	0.86	6.67	**-12.99**	23.96
5	10.46	-0.41	-3.82	-11.62	8.89	-9.09	5.21
6	0.84	-2.07	-6.37	-10	2.22	-21.21	-4.69
7	0.84	-9.13	-2.87	-12.16	-2.22	-24.24	-11.46
8	7.11	6.64	**-12.74**	10.27	-4.44	-20.78	4.17
9	2.51	33.2	-5.41	1.97	-4.44	-21.7	25.57
10	5.36	-6.22	-11.78	4.86	-7.56	-21.13	6.09
11	-3.6	**-11.2**	0	**-11.35**	-5.62	-24.16	**-32.29**
12	2.93	-9.54	-8.6	-7.03	-8.44	-23.38	-10.83
13	-7.53	-22.49	-14.65	-13.51	-8.69	-35.93	-15.52
14	**-15.73**	-12.45	-19.43	-19.73	**-12.78**	-30.91	-14.11
15	-14.74	-11.62	-5.8	-23.24	-6.67	-28.57	-10.16
16	-28.45	-21.58	16.56	-24.32	-6.44	-32.13	-20
17	-31.8	-24.48	3.44	-40.81	-6.44	-40.35	-15.1
18	-9.75	-34.44	-5.41	-38.38	-28.89	-45.02	-29.17
19	-12.72	-39.42	-24.24	-28.11	-20.67	-44.95	-36.98
20	-26.44	-49.12	-28.98	-39.55	-70.22	-48.05	-55.89
21	-25.44	-48.62	-26.75	-38.95	-69.93	-47.19	-55.44
22	-27.66	-50.15	-37.9	-37.12	-69.03	-51.95	-56.78
23	-34.31	-50.64	-39.17	-43.37	-72.12	-57.06	-61.1

Table 2. Behavior of the adsorption and elution capacity of the CB.Hep-1 mAb-immunosorbents. Loss (-), Gain (+) respect to the value of the first purification cycle (100 %). The criterion of instability was ?10 % in at least five consecutive values.

Parameters /LD (mg/mL)	2.67	2.86	3.15	3.58	3.78	P-Va
HBsAg Applied Amount (mg)	3.49 ± 0.05	3.99 ± 0.35	4.05 ± 0.03	4.73 ± 0.10	6.15 ± 0.18	-
HBsAg Adsorbed Amount (mg)	2.18 ± 0.34	2.06 ± 0.56	2.81 ± 0.43	3.38 ± 0.62	3.57 ± 1.49	3.32
HBsAg Eluted Amount (mg)	1.66 ± 0.37	1.65 ± 0.46	2.39 ± 0.28	2.78 ± 0.53	3.10 ± 1.34	6.08
Adsorption Capacity (µg HBsAg/mg mAb)	95.99 ± 14.77	84.85± 22.17	108.05 ± 16.40	101.22 ± 19.93	93.03 ± 28.87	3.89
Elution Capacity (µg HBsAg/mg mAb)	72.95 ± 16.37	67.15 ± 20.05	91.55 ± 10.67	82.42 ± 24.45	80.40 ±27.88	1.74
Adsorption Efficiency (%)	62.44 ± 9.60	57.37 ± 16.16	69.45 ± 10.47	63.81 ± 15.87	59.91 ± 18.32	2.30
Elution Efficiency (%)	75.67 ± 10.37	76.48 ± 11.04	85.33 ± 5.39	78.21 ± 14.20	84.56 ± 7.74	8.57
HBsAg Recovery (%)	47.46 ± 10.63	42.24± 12.79	58.85 ± 6.75	53.28 ± 15.92	51.86 ± 17.20	1.25
mAb Released Amount (ng)	2099.23± 1540.60	885.97 ± 1000.89	1309.70 ± 962.56	2879.57 ± 1479.60	8593.69 ± 8997.82	3.84
Ligand leakage (ng mAb/µg HBsAg)	1.00 ± 0.88	0.45 ± 0.52	0.57 ± 0.43	1.21 ± 0.53	2.61 ± 2.38	1.39

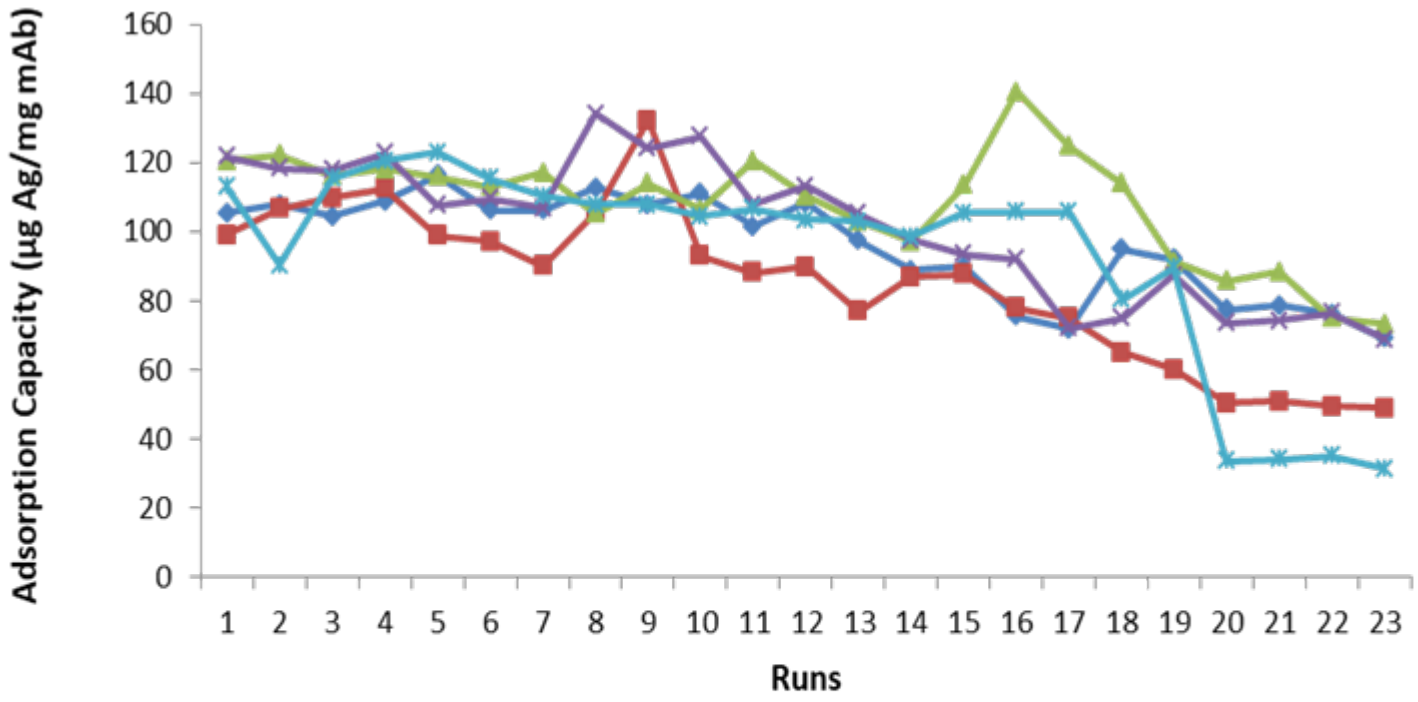
Table 3. Average results of experimental runs of different ligand densities-CB.Hep-1 mAb-immunosorbents applying purified HBsAg samples

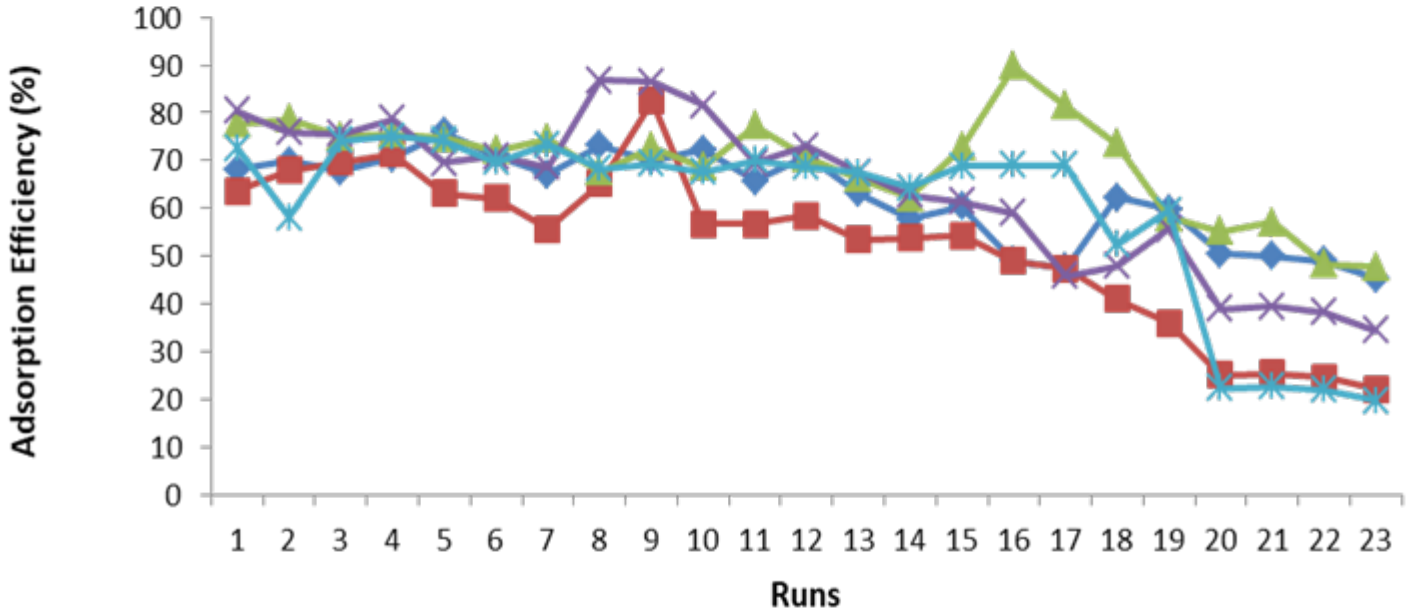
Parameters	Applying Purified HBsAg	Applying Unpurified HBsAg	Kruskal -Wallis P-Values
HBsAg Applied Amount (mg) per run	4.05	5.06	-
HBsAg Adsorbed Amount (mg)-1	3.14	4.16	-
HBsAg Adsorbed Amount (mg)-2	3.18	3.95	-
HBsAg Adsorbed Amount (mg)-3	3.03	4.06	-
HBsAg Adsorbed Amount (mg)-4	3.07	3.53	-
HBsAg Adsorbed Amount (mg)-5	3.02	3.79	-
HBsAg Eluted Amount (mg)-1	2.43	4.75	-
HBsAg Eluted Amount (mg)-2	2.53	4.37	-
HBsAg Eluted Amount (mg)-3	2.41	4.23	-
HBsAg Eluted Amount (mg)-4	2.62	4.61	-
HBsAg Eluted Amount (mg)-5	2.49	4.96	
HBsAg Purity (%)-1 (Monomer +Dimer)	-	90	-
HBsAg Purity (%)-2 (Monomer +Dimer)	-	92	-
HBsAg Purity (%)-3 (Monomer +Dimer)	-	93	-
HBsAg Purity (%)-4 (Monomer +Dimer)	-	94	-
HBsAg Purity (%)-5(Monomer +Dimer)	-	94	-
Carbohydrate content (ng/µg HBsAg)-1	-	8.5	-
Carbohydrate content (ng/µg HBsAg)-2	-	3	-
Carbohydrate content (ng//µg HBsAg)-3	-	6.5	-
Carbohydrate content(ng//µg HBsAg) -4	-	7.5	-
Carbohydrate content (ng//µg HBsAg)-5	-	6.5	-
Lipid content (µg//µg HBsAg)-1	-	1.15	-
Lipid content (µg//µg HBsAg)-2	-	0.16	-
Lipid content (µg//µg HBsAg)-3	-	0.51	-
Lipid content (µg//µg HBsAg)-4	-	0.88	-
Lipid content (µg//µg HBsAg)-5	-	0.65	-
Average HBsAg Adsorbed Amount (mg)	3.09 ± 0.07	3.90 ± 0.24	0.009
Average HBsAg Eluted Amount (mg)	2.51 ± 0.07	4.18 ± 0.43	0.009
Average Adsorption Capacity (µg HBsAg/mg mAb)	118.50 ± 2.74	149.61 ± 0.01	0.009
Average Elution Capacity (µg HBsAg/mg mAb)	96.30± 2.67	160.31 ± 0.02	0.0007
Average Adsorption Efficiency (%)	76.50 ± 1.71	77.00 ± 4.85	0.8
Average Elution Efficiency (%)	78.50 ± 8.89	104.20 ± 8.63	0.009
Average HBsAg Recovery (%)	62.0 ± 1.54	82.53 ± 8.59	0.009
Average HBsAg Purity (%)	-	92.60 ± 1.67	-
Average Carbohydrate content(ng/µg HBsAg)	-	6.40 ± 2.07	-
Average Lipid content(µg//µg HBsAg)	-	0.67 ± 0.37	-
Average mAb Released Amount (ng)	1303.00 ±1168.52	4357.35 ± 3460.52	0.0462
Average Lignnd Leakage (ng mAb/µg HBsAg)	0.57 ± 0.43	1.0 ± 0.80	0.266

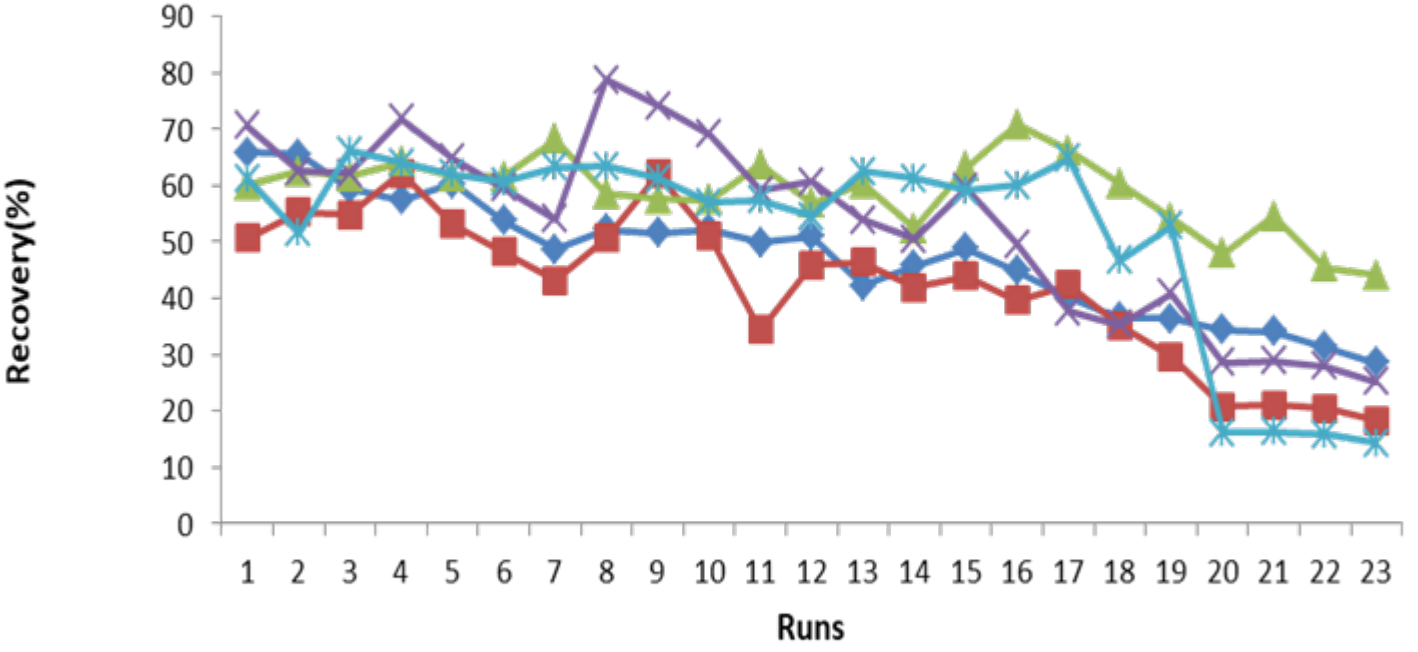
Table 4. Average results of first five purification cycles of the evaluation of 3.15 mg/mL-CB.Hep-1 mAb-immunosorbent applying purified and unpurified HBsAg samples (amount of coupled mAb=26.05 mg)

Indicators	Ligand Density 3.78 mg/mL	Ligand Density 3.15 mg/mL
Cost HBsAg vaccine dose (USD)	0.75	
Product obtained per year (g/year)	440	722.9
Operating cost (USD/year)	4,576.036	6,697.578
Total income (USD/year)	16,5000.000	27,108.750
Number of vaccine batch per year	20	25

Table 5. Comparison of the main economic indicators for CB.Hep-1 mAb- immunosorbents


Adsorption Capacity (µg Ag/mg mAb)
160
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Runs


Adsorption Efficiency (%)
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Runs


Recovery(%)
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Runs


2.67 mg/ml
2.86 mg/ml
3