Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

ELSEVIER

Journal of Biotechnology 96 (2002) 251–258

JOURNAL OF Biotechnology

www.elsevier.com/locate/jbiotec

Chromatographic removal combined with heat, acid and chaotropic inactivation of four model viruses

R. Valdés [a], Neysi Ibarra [a,*], I. Ruibal [b], A. Beldarraín [a], E. Noa [b], N. Herrera [b], R. Alemán [a], S. Padilla [a], J. Garcia [a], M. Pérez [a], R. Morales [c], E. Chong [c], B. Reyes [a], Y. Quiñones [a], A. Agraz [a], L. Herrera [a]

[a] *Center for Genetic Engineering and Biotechnology, Ave 31/158 and 190, PO Box 6162, Havana 10600, Cuba*
[b] *National Reference Center for AIDS, PO Box 23031, Havana, Cuba*
[c] *National Center for Bioreagents, PO Box 6048, Havana, Cuba*

Received 27 August 2001; received in revised form 18 February 2002; accepted 6 March 2002

Abstract

The virus removal of protein A affinity chromatography, inactivation capacity, acid pH and a combination of high temperature with a chaotropic agent was determined in this work. The model viruses studied were sendaivirus, human immunodeficency virus (HIV-IIIb), human poliovirus type-II, human herpesvirus I and canine parvovirus. The protein A affinity chromatography showed a maximum reduction factor of 8 logs in the case of viruses larger than 120 nm size, while for small viruses (18–30 nm) the maximum reduction factor was about 5 logs. Non viral inactivation was observed during the monoclonal antibody elution step. Low pH treatment showed a maximum inactivation factor of 7.1 logs for enveloped viruses. However, a weak inactivation factor (3.4 logs) was obtained for DNA nonenveloped viruses. The combination of high temperature with 3 M KSCN showed a high inactivation factor for all of the viruses studied. The total clearance factor was 23.1, 15.1, 13.6, 20.0 and 16.0 logs for sendaivirus, HIV-IIIb, human poliovirus type-II, human herpesvirus I and canine parvovirus, respectively.

Keywords: Viral validation; Hybridoma; rHBsAg

1. Introduction

Despite rigorous controls being applied to biotechnology production processes, contamination with viruses is a common feature in most of the biological products. Viral contamination of a biological may occur from the source material, e.g. cell banks of animal origin, human blood, human or animal tissues, or as adventitious agents introduced by the production process, e.g. the use of animal sera in mammalian cell culture.

It is for this reason that process validation becomes an important tool for evaluating biopharmaceutical production processes of any mam-

* Corresponding author. Tel.: +53-7-271-6022; fax: +53-7-271-3208.
E-mail address: neysibarra@yahoo.co.uk (N. Ibarra).

0168-1656/02/$ - see front matter
PII: S0168-1656(02)00047-0

malian-derived product. The virus validation should provide a high level of assurance that final product will be free of contaminants.

This kind of study involves spiking the scale down downstream process with a highly infectious virus titer and evaluating the removal/inactivation ability of the whole process or process steps to reduce viruses.

It is not necessary to validate every individual step of a given manufacturing process, only those steps which most likely contribute to inactivate/remove any virus need to be subject to a validation study (Larzul, 1999).

Model or relevant viruses need to be evaluated and these should represent the potential viral contaminants in the starting material (Table 1).

Another critical element into the viral validation process is the validity of the scale down, which can be demonstrated by comparison of process parameters such as pH, temperature and bioseparation variables (e.g. residence time, elution profile, specific activity etc.).

Due to the risk of viral contamination the virus validation study should be performed in a laboratory physically separated from the large-scale production facilities.

In this work we have studied the clearance factor of protein A affinity chromatography, acid pH and a combination of high temperature with a chaotropic agent for the production of a recombinant hepatitis B virus vaccine.

2. Materials and methods

2.1. Monoclonal antibody CB.Hep-1

The monoclonal antibody (Mab) CB.Hep-1 is an IgG_{2b}, specific for the 'a' determinant of the recombinant hepatitis B surface antigen (rHBsAg) (Fernández de Cossío et al., 1997). This Mab is used as immunoligand in the downstream purification process of rHBsAg, which is employed for a commercially available recombinant hepatitis B virus vaccine (HeberBiovac HB™, Heber Biotec

Table 1
Main viral agents potentially present in murine hybridomas, mice and rats

Virus	Genome	Envelope	Size (nm)	Target system
Hantaan and related viruses	RNA	Yes	80–160	Multiple
Sendai	RNA	Yes	100–200	Respiratory
Lymphocytic Choriomeningitis	RNA	Yes	60–280	Hemopoietic
Reovirus type 3	RNA	No	75–80	Digestive
Rotavirus	RNA	No	70–75	Digestive
Minute virus of mice	DNA	No	20–25	Multiple
Ectromelia	DNA	No	175–290	Skin and joints
K virus	DNA	No	35–45	Respiratory
Polyomavirus muris 1	DNA	No	45	Multiple
Mouse thymic virus	DNA	Yes	125–165	Digestive
Pneumonia virus of mice	RNA	Yes	80–200	Respiratory
Lactate DH elevating virus	RNA	Yes	50–55	Hemopoietic
Mouse adenovirus	DNA	Yes	70–90	Digestive
Mouse hepatitis	RNA	Yes	80–160	Digestive
Mouse encephalomyelitis	RNA	No	28–30	Central Nervous
Murine cytomegalovirus	DNA	Yes	120–200	Digestive
Toolan	DNA	No	18–30	Multiple
Rat coronavirus/Sialodacryoadenitis	RNA	Yes	60–200	Digestive
Kilham rat virus	DNA	No	18–30	Multiple

Adapted from: Infectious Diseases of Mice and Rats. National Academy Press. Committee on Infectious Disease of Mice and Rats. Institute of Laboratory Animal Resources. Commission on Life Science. National Research Council.

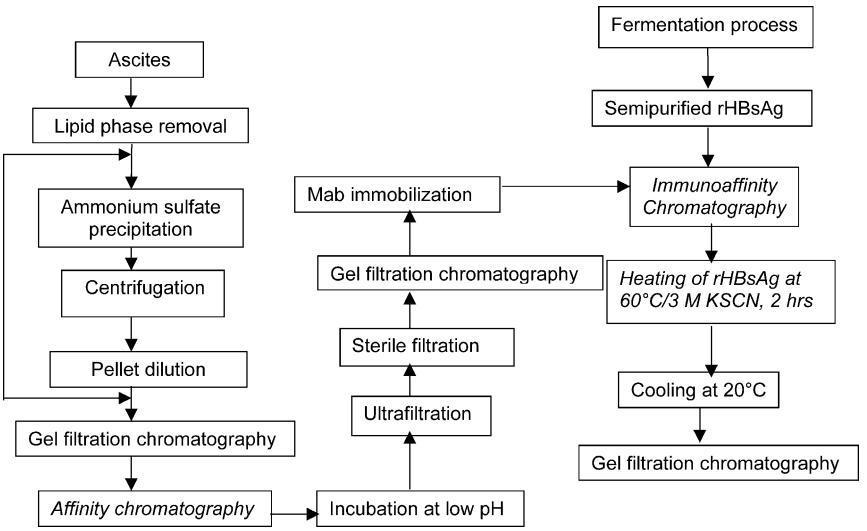

Fig. 1. Purification of the monoclonal antibody CB.Hep-1 and its connection with the rHBsAg purification process.

SA, Cuba) (Pentón et al., 1992; Agraz et al., 1994).

The ascites was obtained from specific pathogens free BALB/c mice, which were previously submitted to rigorous microbiological controls viral infection in the ICLAS Reference Center for Rodents Viruses of Nijmegen, Holland.

The design proposed for the purification of the monoclonal antibody CB.Hep-1 was a combination of several steps including its selective purification by protein A affinity chromatography (Fig. 1).

2.2. Recombinant hepatitis B surface antigen

The rHBsAg was obtained as previously described (Hardy et al., 2000). Briefly, the recombinant *Pichia pastoris* yeast strain was kept under carefully controlled multiplication conditions.

After harvesting, the yeast cells were disrupted (Páez et al., 1993) to recover and purify the rHBsAg by a series of well-established steps. These included acid precipitation (Páez et al., 1993), adsorption/desorption from diatomaceous earth matrix (Agraz et al., 1993) and finally, successive purification through immunoaffinity, ion exchange and gel-filtration chromatographic procedures (Agraz et al., 1993; Pentón et al., 1994).

2.3. Virus clearance studies

The clearance studies were performed in a laboratory separated from the production facilities, scaling down three steps potentially able to remove and/or inactivate viral charges: protein A affinity chromatography, low pH treatment and heating at 60 °C in presence of 3 M KSCN, pH 7.0.

The virus reduction factor (R) was calculated individually and according to the following formula:

$$R = \log\left(\frac{V_1 C_1}{V_2 C_2}\right)$$

where, V_1 and V_2 are the volumes of the starting and post-processing material, respectively, and C_1 and C_2 are the virus concentrations in the starting and post-processing material, respectively. The overall clearance factor was calculated as the sum of each individual removal and inactivation factor (Hageman, 1991).

The model viruses used in this viral validation study (HIV-IIIb), human herpesvirus I, canine parvovirus, human poliovirus type II and sendaivirus (see Table 2) cover a wide range of physical–chemical and structural characteristics of the murine viruses according to the regulatory

Table 2
Characteristics of the model viruses used in the validation study

Virus	Family	Genome	Envelope	Size (nm)	Resistance[a]
Human herpesvirus I	Herpesviridae	DNA	Yes	120	Low
Canine parvovirus	Parvoviridae	DNA	No	18–24	Very high
Human poliovirus type II	Polioviridae	RNA	No	25–30	Medium
HIV-IIIb	Retroviridae	RNA	Yes	100–200	Low
Sendaivirus	Paramyxoviridae	RNA	Yes	100–200	Low

[a] Resistance to physicochemical treatment.

agencies recommendations (CPMP/BWP/268/95, 1996; ICH Guideline, 1997).

2.3.1. Cytopathic effect

The cytopathic effect was determined by the value of the tissue culture infectious dose (D_{50} ml^{-1}), which was calculated following the Reed Muench method (Reed and Muench, 1938). The sensitivity limit of this assay is $10^{1,3}$ $TCID_{50}$ ml^{-1}. Eight experimental determinations per each of the three replies of the reduction and inactivation experiments were used as a criterion in the assays. The cell lines used (HIV negative MT4, Vero and MDK LFKB) were cultured in serum containing medium 5% CO_2 at 37 °C for several days according to the time needed by each virus to express its cytopathic effect. All cell culture materials and reagents used in these assays came from Gibco, Grand Island, USA.

2.3.2. Virus removal in the protein A affinity chromatography step

In Table 3 we show the parameters considered in the scale down of the protein A affinity chromatography step. We determined the purity of the eluted Mab (SDS-PAGE), the process yield and the 'specific activity' of the Mab (ELISA). These were the principal comparative criteria to define process reproducibility. The protocols of these assays have been previously described (Valdés et al., 1994).

Fifty ml of semipurified material was individually loaded with viruses and applied into protein A affinity column (2.5 ml) (Amersham-Pharmacia Biotech, Upssala, Sweden) packed in a c 9/10 column, previously equilibrated with 150 mM phosphate buffer, pH 8.0. Subsequently the matrix was washed with the same buffer and the Mab CB.Hep-1 was eluted with 100 mM citric acid, pH 3.0. The samples were neutralized by addition of 2 M tris before measuring the virus cytopathic effect.

Depending on the maximum titer obtained for each virus we applied different quantities of viruses to the above mentioned column for virus removal evaluation. The initial titer was 8.4, 5.7, 7.5, 10.2 and 9.7 for sendaivirus, HIV-IIIb, human herpesvirus I, human poliovirus type II and canine parvovirus, respectively.

2.3.3. Virus inactivation during incubation at low pH

The viral inactivation at low pH study was carried out by incubating the samples of Mab CBV.Hep-1 containing virus at 4 °C for 7 h in 100 mM citric acid, pH 3.0 and taking samples

Table 3
Parameters considered in the scale down of the Mab CB.Hep-1 production process

Parameters	Large scale	Laboratory scale
Initial volume	5–6 l	50 ml
Linear flow rate	42 cm h^{-1}	42 cm h^{-1}
Column capacity	20 mg IgG ml^{-1}	20 mg IgG ml^{-1}
High	5 cm	5 cm
Residence time	0.1 h	0.1 h
IgG applied	90% of column capacity	90% of column capacity
Temperature	4 °C	4 °C
Adsorption buffer	150 mM phosphate, pH 8.0	150 mM phosphate, pH 8.0
Elution buffer	100 mM citric acid, pH 3.0	100 mM citric acid, pH 3.0

Table 4
Efficiency of the protein A affinity chromatography scale down

Scale	Yield (mg ml^{-1})	Purity (%)	SA[a] (%)
Large scale (20 batches)	1.5	>95	70–100
Laboratory scale (five replicates)	1.6	98	100

[a] Specific activity (SA): Specific activity of Mab CB.Hep-1 ELISA/total proteins.
These values represent the mean of three independent determinations

every hour. The samples were neutralized by addition of 2 M tris to check the cytopathic effect for each virus. The initial titer was 8.0, 5.8, 9.0, 11 and 9.8 for sendaivirus, HIV-IIIb, human herpesvirus I, human poliovirus type II and canine parvovirus, respectively.

2.3.4. Virus inactivation during heating at 60 °C

Each virus model was inoculated in 1 ml of the eluted antigen and was incubated at 60 °C for 2 h. After this, temperature was reduced to 20 °C and the cytopathic effect was measured for each virus. The initial titer was 7.5, 5.5, 8.0, 10 and 8.5 for sendaivirus, HIV-IIIb, human herpesvirus I, human poliovirus type II and canine parvovirus, respectively.

3. Results and discussion

3.1. Virus removal in the protein A affinity chromatography step

We measured viruses by detecting their cytopathic effects (including parvo and poliovirus). This method has the disadvantage that it is culture specific-infectivity and/or replication and the effect depends on the specific virus strain; but it is quantitative, has a broad range of specificity and can detect low levels of viral contamination.

As is shown in Table 4 the process yield, purity and specific activity of the Mab obtained at laboratory scale were similar to the results obtained at large scale.

We evaluated the ability of the protein A affinity chromatography to remove the model viruses previously showed in Table 2. The distribution of the viral amounts onto the column is shown in Table 5.

Due to the specific interaction between protein A and Mab, the matrix was able to remove 86% for sendaivirus, 93% for HIV-IIIb and 97.3% for human herpesvirus I of the respective initial viral charges. It had been hypothesized that charged regions of the viral envelope would interact with protein A media. These high reduction factors coincide with results reported by other authors (Darling and Spaltro, 1996).

Additionally nonenveloped viruses behaved differently than the enveloped viruses. The observed reduction factor was about 71 and 60.1% for human poliovirus type II and canine parvovirus, respectively.

The behavior of nonenveloped viruses can not be explained solely by the hypothesis that certain kind of viruses could interact with the matrix by mean of electrostatic binding of positive charges present in viral enveloped glycoproteins. These results seem to suggest that the chromatographic matrix could also function as a gel filtration matrix. This is possible, because viruses ranging from 18 to 30 nm are trapped in the Sepharose CL-4B and consequently a few viral particles may not be easily removed from the inner of the solid phase. The increase in the quantity of washing buffer prior the elution step to differentiate any unspecific elution from the Mab elution could solve this problem.

In addition, we did not find inactivation of any virus into the chromatographic column. That is an important observation, because it forces us to use acid pH as an inactivating agent after the protein A affinity chromatography. As a consequence, the removal and inactivation factors obtained in the affinity and acid pH steps, respectively, can be analyzed individually, because they work under different mechanisms, which in-

crease the cumulative clearance factor of the whole purification process. However, a longer residence time (more than 7 min) could favor the inactivation process inside the column, allowing both processes (removal and inactivation) to be considered as individual steps in terms of the cumulative process clearance factor.

3.2. Virus inactivation during incubation at low pH

Considering the results obtained for human poliovirus type II and canine parvovirus and also because, no single chromatographic step should be considered sufficient as its own, an additional inactivation step was included. We included the low pH condition, which occurs as part of the purification process, during the elution step (see Fig. 1).

As Fig. 2 (I-A, II-A) illustrates almost all the initial charge of enveloped viruses was completely inactivated by pH 3.0, 75% of inactivation factor was obtained for canine parvovirus and no inactivation was demonstrated for human poliovirus type II after 7 h.

The difference observed in resistance between viruses could be partially explained, because of the presence of the viral envelope.

3.3. Virus inactivation during heating at 60 °C

As it was previously mentioned the purified Mab CB.Hep-1 is used during the immunopurification of rHBsAg, which is eluted from immunoaffinity column, by using 3 M KSCN.

The HBsAg that is initially liberated from yeast cells is a non-disulfide-bonded aggregate of monomer subunits. This aggregated can be converted into fully disulfide-bonded particles that resemble the natural HBsAg by treatment with 3 M KSCN, which is suggested to facilitate exchange (intrachain to interchain) within already oxidized HBsAg polypeptides (Wampler et al., 1985), intrachain and interchain disulfide linkages between dimmers and higher multimers are known to be responsible for stabilizing the correct three-dimensional structure of highly immunogenic HBsAg particles.

As the stability of rHBsAg is increased at high concentration of 3 M KSCN, we combined this condition with high temperature (60 °C) and virus inactivation was analyzed using a phase exponential decay model.

Taking into account this process design, we carried out the spiking experiments by infecting samples with viral charges before heating them and then we evaluated viral reduction after sample desalting. As Fig. 2 (I-B, II-B) shows that 100% of the viral charges were inactivated under this condition during 60 min.

4. Conclusions

Considering these results it is possible to conclude that the protein A affinity chromatography followed by a low pH treatment is able to reduce 16 logs for enveloped viruses. However, no more than 7.7 logs was the maximum clearance factor showed by these two steps for nonenveloped viruses. Human poliovirus type II was not inactivated by citric acid, pH 3.0. The heat treatment is an additional safety level, of-

Table 5
Distribution of the viral charges onto the protein A affinity chromatographic column

Virus type	Initial titer (log)	Non bound titer (LRV)	Elution titer (LRV)
Sendaivirus	8.4	7.2	0
HIV-IIIb	5.7	5.3	0
Human herpesvirus I	7.5	7.3	0
Human poliovirus type II	10.2	7.4	5.2
Canine parvovirus	9.7	5.9	4.4

LRV, log reduction value. The sensitivity limit of the assay is $10^{1.3}$ $TCID_{50}$ ml^{-1}.

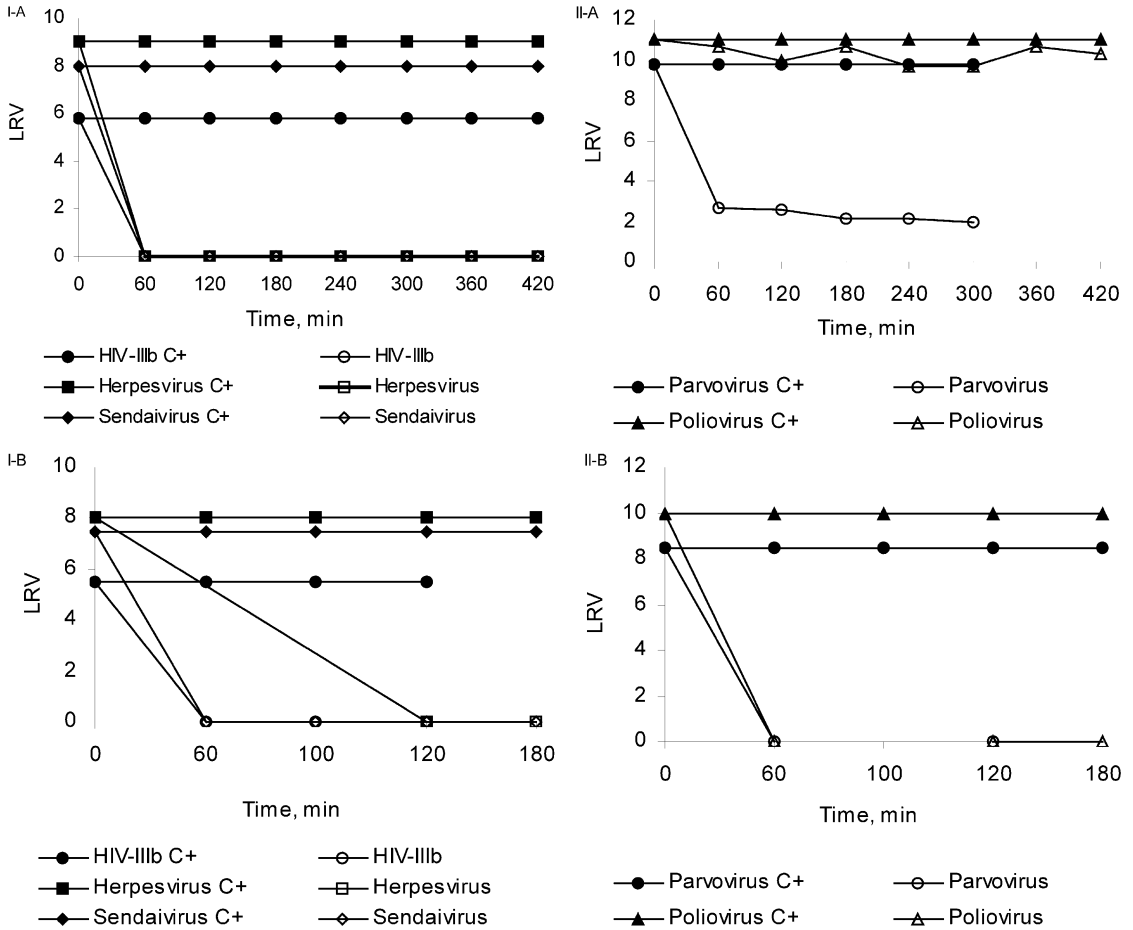

Fig. 2. Virus inactivation curves at low pH (A) and under heat treatment (B) using enveloped (I-A and I-B) and non-enveloped viruses (II-A and II-B), respectively. Positive control (C +): Virus under non-inactivation condition at 4 °C. These values represent the mean of three replicates. LRV. Log reduction value.

fering a robust reduction factor to the production of the Cuban hepatitis B virus vaccine.

Acknowledgements

The authors thank Dr Mac Wainberger for providing us the MT4 cell line and the human immunodeficiency virus (HIV-IIIb). We also thank to the Veterinarian Institute of Hungary for providing us the human herpesvirus I and to the British Institute of Biological Standards and Control for providing us the human poliovirus II. Finally our grateful thanks to Dr Pilar Rodríguez for her technical assistance.

References

Agraz, A., Quiñones, Y., Expósito, N., Breña, F., Madruga, Y., Pentón, E., Herrera, L., 1993. Adsorption–desorption of recombinant hepatitis B surface antigen (r-HBsAg) from *P. pastoris* on diatomaceous earth matrix: optimization of parameters for purification. Biotechnol. Bioeng. 42, 1238–1244.

Agraz, A., Duarte, C.A., Costa, L., Pérez, L., Páez, R., Pujol, V., Fontirrochi, G.J., 1994. Immunoaffinity purification of recombinant hepatitis B surface antigen from yeast using a monoclonal antibody. J. Chromatogr. A 672, 25–33.

The European Agency for the Evaluation of Medical Products, Human Medicines Evaluation Unit-CPMP Biotechnology Working Party, 'Note for Guidance on Virus Validation Studies: the Design, Contribution, and Interpretation of Studies Validating the Inactivation of Studies

Validating the Inactivation and Removal of Viruses,' (CPMP/BWP/268/95 Final Version 2, Canary Wharf, London, 1996).

Darling, A.J., Spaltro, J.J., 1996. Process validation for virus removal. Considerations for design of process studies and viral assays. Biopharm 10, 42–50.

Fernández de Cossío, M.E., Díaz, T., Galván, A., Valdés, R., Gonzalez, E., Ayala, M., Díaz, J., Bestagno, M., Burrone, O., Gavilondo, J., 1997. Antigen recognition characteristics and comparative performance in immunoaffinity purification of two monoclonal antibodies specific for the hepatitis B virus surface antigen. J. Biotechnol. 56, 69–80.

Hageman, T.C., 1991. An analysis of clearance factor measurements performed by spiking experiments. Biopharm 4, 39–42.

Hardy, E., Martinez, E., Diago, D., Diaz, R., Gonzalez, D., Herrera, L., 2000. Large-scale production of recombinant hepatitis B surface antigen from *Pichia pastoris*. J. Biotechnol. 17, 157–167.

ICH Guideline. Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin. March 1997.

Larzul, D., 1999. Viral validation design of a manufacturing process. Dev. Biol. Standardization 99, 139–150.

Páez, R., Agraz, A., Herrera, L., 1993. The recovery of hepatitis B virus surface antigen (HBsAg) from recombinant *P. pastoris* strain, disruption and precipitation studies. Acta Biotechnol. 13, 117–122.

Pentón, E., Herrera, L., Muzio, V., Ramirez, V., García, A., Duarte, C., Ruiz, C., Izquierdo, M., Pérez, L., Fontirrochi, G., González, M., Nazabal, M., Beldarrain, A., Padrón, G., García, J., de la Riva, G., Santiago, A., Ayan, F., Páez, R., Agraz, A., Díaz, R., Quiñones, Y., 1992. Method for obtaining recombinant surface antigen of hepatitis B virus of higher immunogenic capacity and use there of in vaccine preparation. Eur. Pat. Apl. 480, 525.

Pentón, E., Muzio, V., Griego-González, M., 1994. The hepatitis B virus (HBV) infection and its prevention by a recombinant—DNA viral surface antigen (rec-HBsAg) vaccine. Biotecnol. Aplicada 11, 1–11.

Reed, L.J., Muench, H., 1938. A simple method for estimating fifty percent endpoints. Am. J. Hyg. 27, 439–497.

Valdés, R., Leyva, J.L., González, E., Mainet, D., Costa, L., 1994. Caracterización de Anticuerpos Monoclonales contra el Antígeno de Superfície del Virus de la Hepatitis B. Biotecnol. Aplicada 11, 219–224.

Wampler, D.E., Lehman, E.D., Boger, J., McAleer, W.J., Scolnick, E.M., 1985. Multiple chemical forms of hepatitis B surface antigen produced in yeast. Proc. Natl. Acad. Sci. USA 82, 6830–6834.