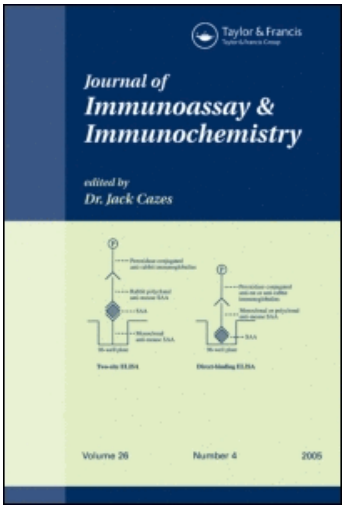

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Online publication date: 05 February 2002

To cite this Article Abrahantes, Maria del Carmen , Reyes, Biunayki , Reyes, Jesus , Sosa, Raudel , González, Ernesto M. , Vega, Maribel , Valdés, Rodolfo and Martínez, Eduardo(2002) 'PROCUREMENT OF REFERENCE MATERIALS AGAINST RECOMBINANT HBsAG SURFACE ANTIGEN', Journal of Immunoassay and Immunochemistry, 23: 2, 229 — 244
To link to this Article: DOI: 10.1081/IAS-120003663
URL: http://dx.doi.org/10.1081/IAS-120003663

J. IMMUNOASSAY & IMMUNOCHEMISTRY, 23(2), 229–244 (2002)

PROCUREMENT OF REFERENCE MATERIALS AGAINST RECOMBINANT HBsAg SURFACE ANTIGEN

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ABSTRACT

Mabs against HBsAg have been used for structural analyses, development of diagnostic tests, and for antigen immunopurification. Resultant products obtained from current methods of genetic recombination demand reference materials to test their potency, identity, purity, and the biological and immunological specific activity corresponding to their manufacturing processes. In this paper, we present a method for the qualitative and quantitative characterisation of CB.Hep-1 and CB.Hep-4 RM and their stability, in real time, with the required quality to be used as primary reference materials. Among the criteria applied in this study, we considered

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Mab-specific concentration through ELISA, purity, and total proteins by various methods, quantification of antigen recognition capacity of Mabs through the Ag-Mab absolute-recognition method. Sterility, homogeneity, stability, subclass, and isoelectric focusing were used in the characterisation of the reference materials.

Abbreviations: Igs, Immunoglobulins; Ag, Antigen; Mabs, Monoclonal antibodies; HBsAg, Hepatitis B surface antigen virus; CB.Hep-1 and CB.Hep-4, Mabs anti-HBsAg; IgG_{2b}, Ig class G and subclass 2b; r-HBsAg, Recombinant HBsAg; RM, Reference material; RI, Radial immunodiffusion

INTRODUCTION

Mabs against HBsAg have been used for structural analysis, development of diagnostic tests, and antigen immunopurification.(1)

CB.Hep-1 is a mouse IgG_{2b} Mab, specific for the "a" determinant of HBsAg (2,3) currently employed as immunoligand in the purification of the r-HBsAg obtained from yeast for the formulation of a commercial anti-hepatitis B virus vaccine (HeberBiovac HBTM, Heber Biotec S.A, Cuba).(4,5) CB.Hep-4 mouse IgG_1 Mab is a relevant candidate for the substitution of CB.Hep-1 as immunoaffinity ligand, due to its 4-fold yield of Mab in ascites (3) and 2-fold yield in hollow fiber bioreactor.(6)

Reference materials (RM) are substances in which one or more properties are sufficiently well established to be used in the calibration of equipment and in the evaluation of a measuring method used to assign values to other materials (ISO Guide 35). They store and transfer the information of the value of a given property in order to standardise measurable quantities. The final products obtained from current genetic recombination methods demand RM to test not only their potency, but also their identity, purity, and specific concentration corresponding to their production process.(7,8) At present, an immunoglobulin type international RM capable of recognising HBsAg does not exist, making it difficult to standardise the different analytical techniques mentioned above for such types of Igs.

We have developed a procedure capable of originating primary RMs of different IgG types, mainly based on absolute methods that could be the basis for the development of primary RMs in future research. Purification and characterisation of CB.Hep-1 and CB.Hep-4 Mabs with the required quality to be used as primary RM are shown in this work.

EXPERIMENTAL

Mab Purification

Radial immunodiffusion (9) to identify contaminant Igs and define the purification method was performed to ascitic fluid rich in CB.Hep-1 and CB.Hep-4 Mabs obtained from the inoculation of such hybridomas in BALB/c mice. Anti Igs mouse sera IgG_1, IgG_{2a}, IgG_{2b}, and IgM prepared in rabbits (ICN Biomedicals, Inc) were used.

As a first purification step, an affinity chromatography using Protein A Sepharose CL 4B,(10,11) having a discontinuous pH gradient, was used for absorption and elution with a linear flow of 40 cm/h (see Table 1). Eluates were neutralized by *Tris* Buffer 2 M. Fractions corresponding to target Mabs were submitted to molecular exclusion chromatography with Superose Preparative 12 (12) matrix, and a linear flow of 4 cm/h (Table 1). The resulting product was filtered under sterile conditions and stored in 100 μL aliquots in 2.5 mL glass bulbs at −20°C.

Qualitative Characterization

CB.Hep-1 and CB.Hep-4 were analysed by RI and isoelectric focusing.(13) To determine the purity of the obtained products, two methods were used: electrophoresis in SDS-PAGE (14) and high-performance size-exclusion liquid chromatography (HPLC-SEC) using

Table 1. Purification of CB.Hep-1 and CB.Hep-4 Mabs

Ascites	C-1 Chromatography		C-2 Chromatography	Preservation Buffer
	Absorption Buffer	Elution Buffer	Run Buffer	
CB.Hep-1	A	D, E, and F	H	H
CB.Hep-4	B	C, D, and F	H	H

Buffer A—PBS pH 8.0; B—1.5 M Glicine/3 M sodium chloride, pH 8.9; C, D, E, F—0.1 M citric acid–sodium hydroxide, pH 6.0, pH 5.0, pH 4.0, and pH 3.0, respectively; G—2 M *Tris* buffer; eluates were neutralized with buffer G; H—20 mM *Tris*/150 mM sodium chloride, pH 7.6.

C-1→ Affinity chromatography using protein A Sepharose CL-4B, with discontinuous pH gradient.

C-2→ Chromatography of molecular exclusion with preparative 12 Superose (Pharmacia) matrix.

TSK 63,000 PW + GuarC and, in the mobile phase of the isocratic chromatography, we used a phosphate buffer containing NaCl 0.25 mol/L, KH_2PO_4 1.7 mmol/L, Na_2HPO_4 7.9 mmol/L, and KCl.

Quantitative Characterisation by Absolute Methods

To assign the quantitative value to antibodies, the Kjeldahl absolute method (15) was applied to the determination of total proteins.

To obtain absolute value of the IgGs capable of recognising HBsAg, this antigen was immobilised. The recombinant HBsAg was dialysed for 12 h at 4°C with buffer 0.1 M $NaHCO_3/NaCO_3$, 0.5 M NaCl, pH 9.0. It was set in contact with BrCN-Sepharose gel which was previously equilibrated with the same dialysis buffer for 2 h at room temperature (24°C) and gently stirred. The rest of the protocol was performed according to Stults et al.(16) Gel capacity was also determined by using 1 mL of the gel with the antigen already immobilised in a column of 1 cm diameter, employing a flow of 50 cm/h and, as absorption buffer of target anti-bodies, a 20 mM *Tris* HCL, 0.5 M NaCl, 3 mM EDTA, 3 M KSCN, pH 7.2, buffer.

Once these parameters had been defined, we proceeded to perform absorption experiments, for which 1 ml of gel with immobilised HBsAg was packed into two columns 1 cm diameter, using a 50 cm/h flow, and the gel was equilibrated with 20 mM *Tris* HCl, 0.5 M NaCl, 3 mM EDTA, pH 7. T samples were 10-fold diluted in the absorption buffer and applied at 25 mL/h. The matrices were washed until reaching the base line and eluted with 20 mM *Tris* HCl buffer, 0.5 M NaCl, 3 mM EDTA, 3 M KSCN, pH 7.2. Samples were taken at different times from its application to the total wash of the matrixes. Two replicates of this absorption experiment were carried out.

Procurement of a Compound Labelled with a Radioactive Isotope

As CB.Hep-1 presented purity antecedents, an experiment to measure its purity through a more sensitive method was performed. A sheep was immunised with CB.Hep-1 Mab. The antiserum obtained was immunopurified using a matrix with immobilised target Mabs. Absorption was performed with PBS pH 7.2 buffer at a rate of 20 cm/h, and eluted with 0.2 M Glycine, pH 2.8. Eluates were neutralised using 2 M *Tris* buffer. The change of buffer was made with 20 mM *Tris*, 150 mM NaCl, pH 7.6, by gel filtration on Sephadex G-25.

The resulting polyclonal was marked with I^{125}, using optimised chloramine T methods: 1.5 mCi NaI^{125} and 30 μL chloramine T (2 mg/mL) were

added to 10 μL of sheep polyclonal at a concentration of 10 mg/mL. It was gently stirred for 60 s. The radioactive isotope-labelled protein was purified through an alumina-blocked PD-10 column G 25 matrix. The buffer used was phosphate–saline buffer (PBS), pH 7.2, and the flow rate was 1 mL/min.

CB.Hep-1 Mab electrophoresis was performed under non-reduced conditions.(14) An autoradiography, using the radio-marked polyclonal, was carried out with conventional methods.

Quantitative Characterisation by Relative Methods

Quantification of total proteins was achieved by the Lowry method, (17) and these results were used to determine homogeneity.

Homogeneity

The homogeneity was defined by quantification of total proteins using the Lowry method. Each bulk was quantified using independent assays and, in every one of them, two replications were made. Variance between bulbs was calculated ($V_1 = DS_1^2$) and the variance of the method ($V_2 = VS_2^2$) was determined during the validation by the Lowry technique. $F_{\text{experimental}}$ (V_1/V_2) was calculated, as well as the associate probability to this value.(18) The criterion of homogeneity applied was a probability higher than 0.05.

Stability Study of Mab in Real Time

Samples were stored at -20°C in 20 mM *Tris* buffer, 150 mM NaCl, pH 7.6 ± 0.2. Six months later, the specific activity was determined as concentration of CB.Hep-1 (ELISA)/concentration of total proteins (Lowry). After this analysis, the testing frequency was every three months for two years. The stability of CB.Hep-4 was analysed each two months for two years by using ELISA. Linear regression was performed to different dots in time. Its slope (m) was determined. A hypothesis test null to slope was made.

Sterility Test

The sterility test was done using the method proposed by the WHO, 1973 (19) and the U.S.P., 1995.(20)

ELISA to Determine CB.Hep-1 and CB.Hep-4 Using Our Own RM

Plates (Costar) were coated (100 μL/well) with a r-HBsAg optimal concentration (10 μg in 0.1 M sodium carbonate-bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. The plates were washed with PBS. A volume of the 100 μL standards, controls, and purified antibody, diluted in 0.15 M PBS, 0.5% (w/v) non-fat milk +0.05% Tween 20, were added to the plates and the plates were incubated for 2 h at 37°C. The plates were washed, and 100 μL of peroxidase-conjugated mouse anti IgG goat polyclonal antibodies (21) was added to each well diluted at optimal concentration (1 : 20 000) and the plates were incubated for 1 h at 37°C. The reaction was developed using 100 μL/well of 0.5 mg/mL *o*-phenylenediamine (OPD) and 0.003% hydrogen peroxide (H_2O_2) in 0.091 M citrate buffer, pH 5.0. After 20 min, the reaction was stopped with 50 μL/well of 1.25 M H_2SO_4. The absorbancy was measured in a Microtek ELISA reader using a 492 nm filter.

The linearity study was performed using different concentrations of samples of interest (CB.Hep-1: 50; 25; 12.5; 6.25; 3.12 ng/mL and CB.Hep-4: 8; 6.25; 4; 3.12; 2; 1.56; 1 ng/mL) and every spot was 3-fold analysed. Calibration curves with different concentrations of the sample and the response measured in absorbancy were used. The least square method was applied to obtain the function describing a linear model.

The determination coefficient (r^2) and probability associated to *F* Snedecor (22) were determined by Microsoft Excel software (Microsoft Corporation, USA).

RESULTS

Mab Purification

In order to identify possible Igs of the ascites rich in target Mabs (CB.Hep-1 and CB.Hep-4), RI was done to both biological materials. IgM, IgG_{2a}, and IgG_{2b} subclasses were detected in the RI assay of CB.Hep-1, while the presence of IgG_1 and IgM was shown in that of CB.Hep-4. The IgGs were separated with affinity chromatography. The IgM was suppressed by molecular exclusion chromatography. HPLC–SEC was performed to analyse the purity. The chromatographic result was obtained, reproduced, and processed by software Bio-Crom (CGEB, Cuba) and registered by computer.(23) Purity of the final product determined by the above method was 96.5% (SD 0.54) and 97.1% (SD 0.10) for CB.Hep-1 and CB.Hep-4, respectively (Figure 1). Purity by electrophoresis

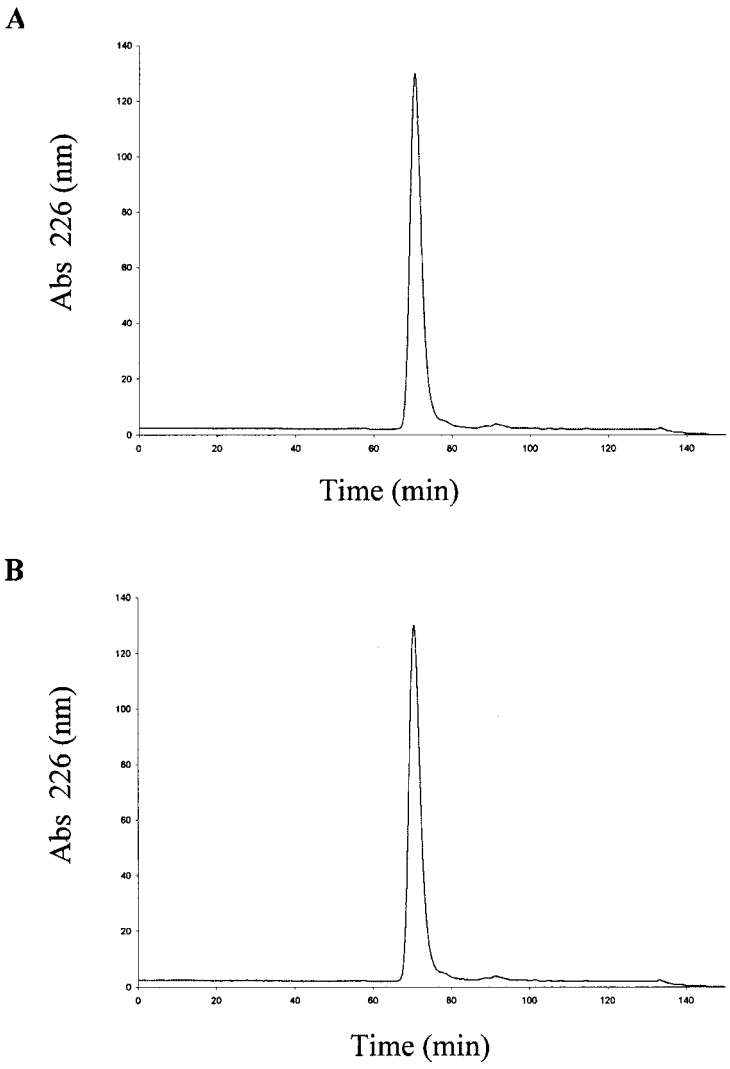

Figure 1. High performance size exclusion liquid chromatography (HPLC-SEC) using TSK 63000 PW + GuarC ($n = 12$). Chromatograms were obtained, reproduced, and processed by Bio-Crom Software (Havana, Cuba). The isocratic chromatographic mobile phase was prepared with a phosphate buffer containing 0.25 mol/L NaCl, 1.7 mmol/L KH_2PO_4, 7.9 mmol/L Na_2HPO_4, and 2.7 mmol/L KCl, pH 7.0. The column effluent was continuously monitored by absorbance at 226 nm and a flow rate of 0.2 mL/min. Purity of the final product, determined by chromatography, was 96.5% (SD 0.54) and 97.1% (SD 0.10) for CB.Hep-1 and CB.Hep-4, respectively.

in SDS-PAGE was 96.0% (SD 1.4) and 97.2% (SD 1.61) for CB.Hep-1 and CB.Hep-4, respectively (Table 2). No significant differences in the results of both techniques were found ($p > 0.05$).

Quantitative Characterisation

The Kjeldahl absolute method and the Lowry relative method were used for protein determinations. The results were 0.889 mg/mL (SD 0.04) and 1.778 mg/mL (SD 0.06) for CB.Hep-1 and CB-Hep-4, respectively, with the Kjeldahl method. The results with the Lowry method were 0.881 mg/mL (SD 0.038) and 1.876 mg/mL (SD 0.058) for CB.Hep-1 and CB-Hep-4, respectively (Table 3).

Absorption and Specific Elution of Antibodies (Immunopurification)

The results of the HBsAg immobilisation process were 0.25 mg/mL of gel ligand density, 34% of immobilisation, and 0.24 mg/mL of gel absorption capacity. Fifteen percent of the gel capacity from CB.Hep-1 and eight percent from CB.Hep-4, equivalent to 0.036 mg and 0.021 mg, respectively, were applied. During the sample absorption, no significant protein detection signals in the collected volume were found, nor in the protein determination by absorbancy at 280 nm, using a molecular extinction coefficient of 1.4 (Figure 2).

No significant absorption peaks were spotted during the application of the sample to the immunoabsorbent, which suggests that all proteins presented antigen recognition capacity and that an absolute value determined by Kjeldahl could be assigned.

Compound Marked with Radioactive Isotopes

The procedure to generate the compound marked with a radioactive isotope had an efficiency of 78.3%, a specific activity of 11.7 μCi/μg, purity 97%, and a radioactive isotope molecule by protein molecule relation of 0.84.

Autoradiography results showed that the seemingly contaminant band was somewhat lower than the wider one (Figure 3) and was readily recognised by the polyclonal-marked I^{125}, suggesting the presence of an isoform of the CB.Hep-1 with a different electrophoretic mobility corresponding to the IgG_{2b} subclass.

Table 2. Results of Reference Material Characterization

Parameter	Method	Results	
		CB Hep.1	CB Hep.4
Purity	HPLC Filtration gel	$96.53 \pm 0.54\%$	$97.18 \pm 0.10\%$
	SDS-PAGE Electrophoresis	$96.00 \pm 1.40\%$	$97.20 \pm 1.61\%$
Total proteins	Lowry	0.880 ± 0.03 mg/mL	1.870 ± 0.05 mg/mL
	Kjeldahl	0.889 ± 0.04 mg/mL	1.778 ± 0.06 mg/mL
Ag–Mab recognition	Immunoaffinity chromatography	Complete absorption	Complete absorption
Sterility	Direct seeding	Approved	Approved
Homogeneity	Lowry	Fdist(p) > 0.05	Fdist(p) > 0.05
Stability	ELISA	2 years (-20°C)	2 years (-20°C)
Subclass	IR	IgG_{2b}	IgG_1
Isoelectric point	Isoelectric focussing	6.86	6.66

These results show the presence of RM with adequate features to be considered primary and a method responding to the established requirements for this kind of material, which could be conditioned to the specific characteristics of other Mabs needing primary reference material for further studies.

Table 3. Statistical Stability Analysis

Ref. Mat.	Slope	F.Sig	t_{exp}	$t_{(0.05, n-2)}$	Ho: $m=0$
CB.Hep-1	0.005	0.186	1.409	2.201	Accepted
CB.Hep-4	−0.029	0.060	2.107	2.201	Accepted

A hypothesis test, null to the slope of the linear function, describing the behaviour of the different determinations in time of biological activities for CB.Hep-1 and CB.Hep-4. No significant differences exist between the function slope and the 0 value to both reference materials ($t_{theoretical} > t_{exp}$), which suggests that such values are stable at least for two years.

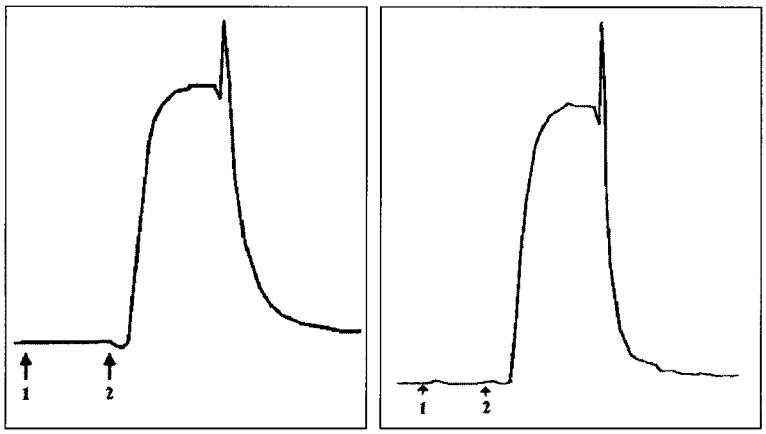

Figure 2. The chromatogram shows the recognition capacity of IgG (A: CB.Hep-1 and B: CB.Hep-4) immunoglobulins with immobilised HBsAg. As we can neatly see, the matrix in both cases absorbs almost the entire sample. 1—Sample application; 2—Elution with 20 nM *Tris* HCl buffer, 0.5 M NaCl, 3 mM EDTA, 3 M KSCN, pH 7.2.

Homogeneity

Each bulb was quantified using independent assays and, in every one of them, two replications were made using the Lowry method. Variance between bulbs (V_1) was calculated, as well as the variance of the method (V_2). F $\text{Snedecor}_{experimental}$ was calculated as V_1/V_2 and also the associated probability to this value was determined. The p associated to F $\text{Snedecor}_{experimental}$ was 0.33 and 0.10 for CB.Hep-1 and CB.Hep-4,

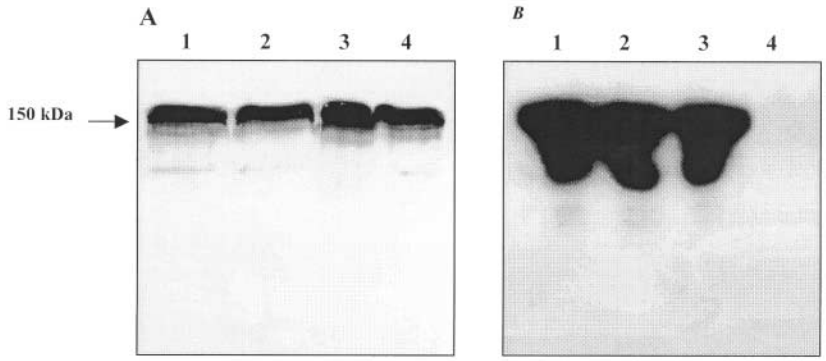

Figure 3. Autoradiography results show that the seemingly contaminant band was somewhat lower than the wider one (Figure 3) and was readily recognized by the polyclonal marked I^{125}, suggesting the presence of an isoform of the CB.Hep-1 with a different electrophoretic mobility corresponding to the IgG_{2b} subclass.

respectively. It was higher than 0.05 for both materials, showing that no significant differences existed between determinations of different bulbs.(17)

Stability

The t_{exp} was 1.40 and 2.10 for CB.Hep-1 and CB.Hep-4, respectively, lower than $t_{0.05,n-2}$ (2.20). In those reference materials, there is no significant difference of values with time, which suggests that such values are stable at least for two years (Table 3).

Linearity

As shown in Figure 4, the "average curve" of the points obtained by plotting the absorbance values against concentration values exhibited linearity in the working concentration range analysed (CB.Hep-1: 50; 25; 12.5; 6.25; 3.12 ng/mL and CB.Hep-4: 8; 6.25; 4; 3.12; 2; 1.56; 1 ng/mL). The calculated *F* Snedecor associated probabilities were 0.06 and 0.13 for CB.Hep-1 and CB.Hep-4, respectively. As it is higher than 0.05, the proposed equation represents experimental data. The determination coefficients was approximately 0.99.

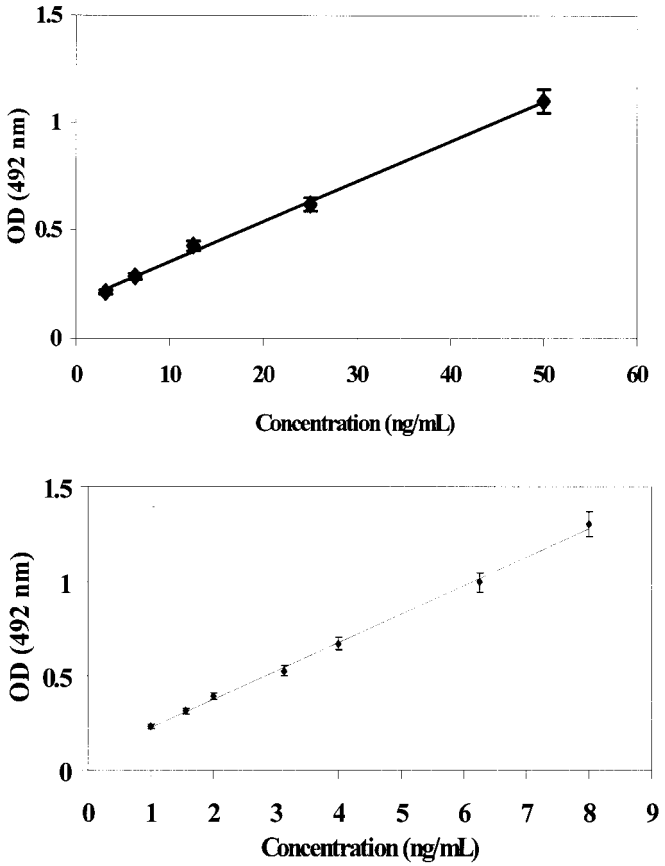

Figure 4. The curve exhibited linearity in the concentration analysed. The calculated *F* Snedecor associated probabilities were 0.06 and 0.13 for CB.Hep-1 (A) and CB.Hep-4 (B), respectively. Above 0.05, the proposed equation is a good representation of experimental data. The determination coefficients were approximately 0.99.

DISCUSSION

Our center produces a commercial anti-hepatitis B virus vaccine (HeberBiovac HBTM, Heber Biotec S.A., Cuba). In the manufacturing processes, in the purification of the r-HBsAg, the Mabs CB.Hep-1 or CB.Hep-4 is employed as immunoligand. Its Mabs also are used in the diagnostic test of HBsAg. However, we do not have international reference materials of IgG murine for the calibration of secondary standards. The

Committee on Biological Standardization reconnoitered the need for reference materials for testing diagnostic kits used for detection of HBsAg at a conference held 11–12 December, 1996, at WHO, Geneva. Due to this need, our laboratory worked in obtaining the reference materials based in a method that could be extended to the obtaining of other reference materials of this type.

IgGs type contaminants present in ascitic fluid were separated by affinity chromatography. CB.Hep-1 obtained in the elution at pH 4, and IgG type contaminants were excluded at pH 5 and pH 3. CB.Hep-4 was detected in the elution at pH 6. These results were similar to those reported by Pharmacia Biotech, in 1994, because CB.Hep-1 is a IgG_{2b} and CB.Hep-4 is a IgG_1. In both preparations, IgM type contaminants were eliminated by molecular exclusion chromatography.

The quality of the product obtained during purification was demonstrated by HPLC-SEC and by electrophoresis in SDS-PAGE. The purity was above 96%, confirming the reproducibility of the results, taking into account that they were performed in different analyses, on different days, and at different labs. It is particularly important to point out that both an absolute and a relative method were used.

The immunopurification process showed absorption of all the reference materials included in the samples. No significant peaks were noticed during the application of the samples to the immunoabsorbent, and proteins were not detected by absorbancy at 280 nm when using a molecular extinction coefficient of 1.4 (Figure 2). These results suggest that all the proteins applied to the immunoabsorbent were capable of recognising the antigen which gave rise to them, and that the absolute value determined by Kjeldahl can be assigned as functionally active antibodies.

Autoradiography results suggest the presence of isoforms of CB.Hep-1 in the final product, with an electrophoretic mobility corresponding to IgG_{2b} subclass.

The study of homogeneity performed showing that p associated to F $\text{Snedecor}_{\text{experimental}}$ was higher than 0.05 for both materials means that no significant differences existed between the determinations of different bulbs and also suggests that aliquots made of RM are homogeneous.

All reagents eventually lose activity when stored at elevated temperatures, and stability shall be checked by an accelerated degradation test. However, accelerated degradation studies are considered to be only a useful rather than an absolute guide to the stability of reference materials maintained by the manufacturer. Therefore, the stability of the reference materials must also be determined for the conditions under which they are stored, in a real-time stability study.(24) The biological materials analysed in this work, CB.Hep-1 and CB.Hep-4, were stored at $-20^{\circ}C$

and their stabilities were checked for two years. The analysis of stability by the graphic method showed no significant difference between the values shown in time, which suggests that they are stable for at least two years (Table 3).

In ELISA assays, the standard curve exhibited linearity in the concentration analysed, as the p associated to F $\text{Snedecor}_{\text{experimental}}$ was higher than 0.05 for our two candidates. That is why the proposed equation is a good representation of experimental data. The determination coefficient was approximately 0.99, showing the adjustment quality to the linear model.

Arnout et al.(25) used it as reference material for a lupus anticoagulant test. Recently, a series of murine monoclonal antibodies were raised against human beta2GPI, used as reference materials. However, in our work, we realised the characterisation of the biological materials (Table 2) with adequate features to be considered primary, and a method bearing the established best requirements for this type of material, which could be conditioned to the specific characteristics of other Mabs needing a primary reference material for further studies. The results (Table 2) show the presence of RMs suitable to be considered as primary.

ACKNOWLEDGMENTS

I thank Lic. José L. Fernández Sierra, whose systematic support made possible the completion of this paper, as well as for the revision of the manuscript.

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Received May 13, 2001
Accepted July 9, 2001
Manuscript 3044