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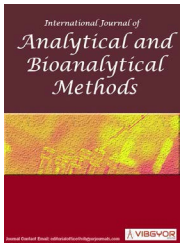
Validation of Two-Tiered Enzyme-Linked Immunosorbent Assay for the Detection of Anti-HeberProt-P® Antibodies



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Carlos Hernández[1#], Maylin Pérez-Bernal[1*#], Emilio Carpio[2], Onel Valdivia[1], Magali Delgado[1], Mabel Izquierdo[3], Ileana Rosales[3] and Enrique Pérez[1]

[1]*Research-Development Department, Center for Genetic Engineering and Biotechnology of Sancti Spiritus, Cuba*

[2]*University of Medical Sciences of Sancti Spiritus, Cuba*

[3]*Quality Control Direction, Center for Genetic Engineering and Biotechnology, Cuba*

[#]*Authors contributed equally*

Abstract

Heberprot-P® is a therapeutic product approved in Cuba for the treatment of diabetic foot ulcer. In spite of its ample clinical use, its immunogenicity in patients has not been evaluated. Regulatory agencies have developed guidelines for the validation of immunoassays designed at the detection of anti-drug antibodies to biologicals. The aim on this work was to validate a method for the detection of binding antibodies in human serum to recombinant human epidermal growth factor (rhEGF), the active ingredient of Heberprot-P®. A two-tiered enzyme-linked immunosorbent assay (ELISA) format was used. First tier was a screening assay, based on immobilized rhEGF and anti-human polyvalent alkaline-phosphatase conjugated as development reagent. Positive samples were then tested with a confirmatory assay which used a competitive soluble rhEGF in solution. As FDA and EMA guidelines recommend, minimum required dilution, quality controls, screening and confirmatory cut points, sensitivity, recovery, precision, specificity, selectivity, robustness and stability of samples were determined. The assay was precise and its sensitivity was calculated to be 214.2 ng/mL. The majority of evaluated parameters fulfilled the figures recommended by current guidelines. The immunoassays described in this work could be reliable tools for the evaluation of immunogenicity of Heberprot-P® in well-designed clinical studies.

Keywords

ADA, ELISA, Recombinant human epidermal growth factor (rhEGF), Validation

Introduction

As a result of the revolution in genetic engineering and biotechnology, a new group of drugs has emerged: Biopharmaceuticals; also known as therapeutic proteins or biologicals. Since the marketed of insulin (Humulin) by Eli Lilly in 1982 as the first drug of this class, a steadily increasing number of biologicals have been approved for human use and they are currently considered as a vital component

***Corresponding author:** *Maylin Pérez-Bernal, Research-Development Department, Center for Genetic Engineering and Biotechnology of Sancti Spiritus, P.O. Box 83, Sancti Spiritus 60200, Cuba*

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of the therapeutic arsenal in modern medicine. Biopharmaceuticals include hormones, antibodies, enzymes, growth factors, among others. The positive impact of these drugs in numerous diseases has been unquestionable, but the very nature of these large molecules has an important drawback: They can be immunogenic in human subjects. Unwanted immune response to therapeutic proteins has the potential to affect its efficacy, safety, pharmacodynamics and pharmacokinetics. The clinical effects of these immune responses in patients range from no measurable (clinically benign) to extremely harmful [1]. Even when both arms (humoral and cellular) of immune response can be activated by these drugs, the presence of anti-drug antibodies (ADA) has been of special concern. Taking this potential risk into consideration, regulatory agencies request that immunogenicity must be assessed as part of the approval process of therapeutic proteins and this information should be included in the in the prescribing information as a subsection of the "adverse reaction" section entitled Immunogenicity [1]. Regarding detection of ADA, regulatory agencies have developed statements and guidelines advising the proper methods and type of assays for the evaluation of unwanted immunogenicity, highlighting measurement and characterization of antibodies [2,3].

Heberprot-P® is a freeze-dried formulation containing recombinant human epidermal growth factor (rhEGF), as active ingredient. This therapeutic protein product has been approved in Cuba for the treatment of diabetic foot ulcers (DFU). The randomized clinical trial, on which its approval is based, showed that intra-lesional injection of recombinant human EGF increase granulation and healing in advanced DFU [4]. Thousands patients have been treated with Heberprot-P® during the last decade with positive therapeutic effects and favorable safety profile. A recent meta-analysis and systematic review on the use of rhEGF (including but not only Heberprot-P®) for the treatment of DFU concluded that "the available evidence supports the use of rhEGF in facilitating the healing process of DFU" [5].

In spite of its wide use in clinical practice, the immunogenicity of Heberprot-P® and its influence on the efficacy, pharmacokinetics and safety of this drug is unknown. In order to obtain this information a proper method and immunoassays are needed. The aim of this work was to validate an immunoassay for the detection of ADA in serum from patients treated with Heberprot-P®. For this purpose, current guidelines and recommendation from the appropriate regulatory agencies have been followed.

Materials and Methods

Assay procedure

A two-tiered bridging enzyme-linked immunosorbent assay (ELISA) format was selected. All "putative positive" samples from the screening assay (tier 1) were tested in the presence of excess of antigen 1 h at (22-25) °C as inhibition step in the confirmatory assay (tier 2).

Tier 1: 96-well microtiter plates (Thermo Scientific™ Nunc™ Polysorp) with strip plate modules, were coated with 10 µg/mL of antigen (Biological Raw Material of rhEGF, CIGB, Cuba) in 100 µl/well coating buffer (phosphate-buffered saline, pH 7.2) and incubated 1 h at (22-25) °C. Wells were washed four times with 380 µl/well washing buffer diluted 1:25 (WB 25X: 0.292 M Tris-hydroxymethyl-aminomethane, 0.228 M HCl, 3.75 M NaCl and 1.25% Tween-20) and blocked with 350 µl/well blocking buffer (BB: 20 mM Tris-HCl, 1.0 M NaCl and 1% Bovine Serum Albumin) 90 min at 37 °C. The plate was washed once with WB, and 100 µl of serum samples and quality controls (in duplicate), diluted with dilution buffer (DB: 20 mM Tris-HCl, 1.0 M NaCl, 1% bovine serum albumin and 1% Tween-20), were added to wells and incubated 2 h at (22-25) °C. DB was used as blank. Plates were washed four times and 100 µl of anti-human polyvalent alkaline-phosphatase (AP) (Sigma; 1:12000 in DB) was added to each well and incubated 2 h at (22-25) °C. After four washes, 100 µl/well p-nitrophenyl phosphate (1.0 mg/mL) with 1.0 M di-ethanolamine, 0.5 M $MgCl_2$, pH 9.8, was added and incubated 1 h at (22-25) °C, in the dark. Color development was terminated by adding 100 µl/well 0.1 M di-sodium EDTA. Absorbance was measured at 405 nm (A_{405nm}) in a Multiskan plate reader.

Origin of human serum

Individual human serum samples for cut-point determination were obtained from 60 healthy donors. A pool of sera from 20 diabetic patients with foot ulcer, before treatment with Heberprot-P, was used to prepare negative quality control. Positive control for validation and positive quality controls

were prepared from a pool of sera of three patients treated with CimaVax-EGF vaccine [6], with a concentration of 162.4 µg/mL of anti-EGF antibodies. The positive control for validation was diluted with serum from healthy donors, prepared at the minimum required dilution with DB. All sera were stored at -20 °C until use.

Minimum required dilution

To determine the minimum required dilution (MRD), ten serum samples from healthy donors were two-fold serial diluted with DB, ranging from 1:5 to 1:640.

The percent of background signal reduction (%BR) in each sample, in relation with the A_{405nm} from sample diluent (DB), was determined by the formula [7]:

% BR = 100 × (mean A_{405nm} of sample - mean A_{405nm} DB)/mean A_{405nm} DB

The acceptance criterion for MRD was based on %BR ≤ 100% for at least 80% of serum samples.

Recovery

To perform a spike-and-recovery experiment, a known amount of anti-EGF antibodies was added to the sample matrix and to the diluent DB. Dilution curves of anti-EGF antibodies were achieved by serial 1:2 dilutions of positive control, from 30 ng/mL to 0.23 ng/mL. The sample matrix and the DB were the curve diluents. The sample matrix was composed by five individual serum samples from healthy donors or five individual serum samples from patients treated with Heberprot-P®, all diluted 1:100 with DB.

Percent of recovery (%R) was calculated at three concentration points of the curve (15, 3.8 and 0.94 ng/mL), by the formula:

$$\%R = \frac{\text{In - matrix - Concentration (using sample matrix as diluent)}}{\text{In - buffer - Concentration (using DB as diluent)}} \times 100$$

A recovery between 80 and 120% was accepted.

Screening cut-Point

The screening cut-point was established by analyzing 60 individual human serum samples from healthy volunteers, distributed in three subgroups of 20 samples, by two analysts over three different days each one. Negative and positive controls were included in all assays. Every day was performed three ELISAs, following a balanced design [8].

A parametric approach (one sided, 95% confidence level) was selected to calculate the cut-point. Outliers were identified by the Box Plot method and they were removed from each single assay run. Normality of ln-transformed A_{405nm} data were carried out using the Shapiro-Wilk test (α = 0.05).

To decide if a fixed or a floating cut-point could be used, two statistical tests were performed after outlier removal: 1) Variance homogeneity of log-transformed data was analyzed by Levene test (α = 0.05); 2) Single factor ANOVA (α = 0.05) to evaluate if the means of the runs are significantly different or not [8]. A floating cut-point was used for screening, since the variances were homogeny but the means were significantly different. The screening cut-point was defined as: mean A_{405nm} (negative control in clinical assay) × normalization factor. The normalization factor was calculated by using the difference between the parametric cut point (CP = mean (data from pre-study validation) + 1.645 × SD) and the mean of negative control. The standard deviation (SD) was estimated by performing a variance component analysis using restricted maximum likelihood method within the framework of a nested ANOVA.

Confirmatory cut-point

For establishment of confirmatory cut-point, an inhibition step was performed to the samples, prior to place them in the ELISA plates. The same 60 serum samples, distributed in balanced design used for the determination of the screening cut-point, were incubated 1 h at (22-25) °C with 100 µg/mL of antigen and tested along with the corresponding non-inhibited samples, during six days by two analysts (three days per analyst).

The dependent variable used was R, the ratio between A_{405nm} of inhibited sample and A_{405nm} of non-inhibited sample. Data were assessed for outliers as described in the screening cut point. Confirmatory cut-point was determined by non-parametric way (percentile 1 from variable R). It was calculated by the formula:

$CP_{0.99} = 100 - (R \times 100)$

Sensitivity

The sensitivity of the assay was measured by testing a curve with serial 1:2 dilutions of positive control, from 30 ng/mL to 0.23 ng/mL. Each point of the curve had three repetitions into the ELISA plate. The assay was replicated in six days by two analysts, three assays each one.

The concentration of the positive control yielding an assay response at the cut-point was interpolated using a 5PL-fitting model. For determination of a consistent sensitivity value, the sensitivity was calculated with the following equation (aiming for a 5% failure rate):

Sensitivity = mean concentration at cut-point + 2.015 × SD

Variability of means attributable to analyst was analyzed by using data from each run as assay repetition. SD was estimated by performing a variance component analysis using restricted maximum likelihood method within the framework of a random one-way ANOVA.

Precision

The precision of the screening ELISA was achieved by serial 1:2 dilutions of positive control, from 30 ng/mL to 0.23 ng/mL. For the confirmatory ELISA, it was added an additional step of pre-incubation of each dilution point with 100 µg/mL of antigen during 1h at (22-25) °C. The inhibition percentage (%I) was calculated as follows:

%I = 100 - 100 × (A_{405nm} Inhibited point/A_{405nm} Non-inhibited point)

Precision of both ELISAs was assayed for negative control and for high (30 ng/mL), medium (3.75 ng/mL) and low (0.938 ng/mL) concentrations of positive control. The runs were performed over six days by two analysts, three days each one. Each point had two repetitions in two different positions into the ELISA plate.

SD was estimated by a variance component analysis using restricted maximum likelihood method within the framework of a nested ANOVA.

Precision was evaluated as intra-assay (repeatability) and inter-assay (intermediate precision) and it was expressed as percent of coefficient of variation (CV), which should be less than 20%.

In the screening ELISA the dependent variable was ln-transformed A_{405nm} data and the CV was calculated according to the formulas:

Intra-assay precision: CV (%) = $(e^{\text{variance of error}} - 1)^{0.5}$ × 100

Inter-assay precision: CV (%) = $(e^{\sum\text{variance components}} - 1)^{0.5}$ × 100

In the confirmatory ELISA the dependent variable was %I and the CV was calculated as follows:

Intra-assay precision: CV (%) = [(error variance)$^{0.5}$/mean] × 100

Inter-assay precision: CV (%) = [(∑variance components)$^{0.5}$/mean] × 100

Specificity

The evaluation of specificity was done by an inhibition assay, as suggested the USP (2014). The positive control was inhibited with 100 µg/mL of the antigen during 1h at (22-25) °C. The non-inhibited control had PBS instead of the antigen. The valuation was performed using high (30 ng/mL), medium (7.5 ng/mL) and low (1.88 ng/mL) concentrations of positive control. The inhibited and non-inhibited specimens were placed into the ELISA plates, using two variants: Plate coated with the antigen and blocked with BB as described before, and plate without coating but blocked with BB, which was used for background signal correction. Each concentration point of positive control was triplicated into the plates. The percent of inhibition (%I) was calculated as follow:

%I = 100 - 100 × (A_{405nm} inhibited)/(A_{405nm} non-inhibited)

ELISA was considered specific if the mean of the percent of inhibition was close to 100%.

Quality controls confidence intervals

The quality controls (QCs) of the ELISA were prepared as follows:

Negative control (NC): Pool of sera from 20 diabetic patients with foot ulcer before treatment with Heberprot-P.

High positive quality control (HQC) and low positive quality control (LQC) were prepared from positive control. Both were prepared at 100X with a pool of sera from healthy donors. For the ELISA assay, they were diluted 1:100 with DB, to get final concentrations of 20 ng/mL (HQC) and 2.5 ng/mL (LQC).

The acceptance limits of QCs were established by six ELISA assays in different days by two analysts, three assays each one. QCs were triplicated in the ELISA plates. For NC the upper limit of A_{405nm} was determined at 99% confidence by the formula: mean A_{405nm} + t (0.01, DF) x SD. For HQC and LQC the two-sided confidence intervals (99%) were

determined using the mean of the A_{405nm} ratios of HQC/NC and LQC/ND, as dependent variables, by the formula: mean ± t (0.01, DF) × SD.

Selectivity/Interference

Selectivity was evaluated by testing three types of samples: Ten individual hemolytic sera, ten individual lipemic sera and ten individual sera from diabetic patients with foot ulcer. Samples were diluted at MRD and all were spiked with two concentrations of positive control: Low concentration (LC = 2.5 ng/mL) and high concentration (HC = 20 ng/mL). The experimental controls were two pools of sera from healthy donors, spiked with LC and HC of positive control, respectively. Data were measured in duplicates. The percent of recovery was calculated by the formula:

%R = (mean A_{405nm} sample/mean A_{405nm} control) × 100

A recovery between 80 and 120% was accepted.

The EGF interference was evaluated by pre-incubation of increasing amounts of EGF (0.0134, 0.0665, 0.330, 1.64, 8.11, 40.2, 199.6 and 990) ng/mL with 2.5 ng/mL of positive control. The diluent was serum from healthy donors prepared 1:100 with DB. The pre-incubation occurred during 1 h at (22-25) °C, before place it into the ELISA plate. The assay tolerance limit to endogenous EGF was determined by interpolation of A_{405nm} immediately under screening cut point, in a calibration curve adjusted by a logistic regression of five parameters. Data were measured in triplicates.

Robustness

There were made small changes in critical parameters of the ELISA procedure: Variation in ± 5 min the plate incubation times for antigen coating, samples and anti-human polyvalent AP-conjugated; decreasing of substrate concentration from 1.0 mg/mL to 0.8 mg/mL; using bovine serum albumin (BSA) from three suppliers: Sigma, Roche and AppliChem, for the preparation of BB and DB for samples and AP-conjugated; using compacted Thermo Scientific™ PolySorp ELISA plate instead of PolySorp strip plate module. Each treatment was evaluated in triplicates. The robustness was proved by the fulfilling of the QCs confidence intervals (99%).

Stability of samples and quality controls

The origin of samples and QCs was the same, for that reason the stability of both was proved by the fulfilling of the QCs acceptance limits at 99% confidence. Short-term stability (3 and 6 hours at room temperature (22-25) °C and 6 days at (2-8) °C) and freeze-thaw stability (five freeze-thaw cycles), were tested by using the QCs. For the evaluation of freeze-thaw stability, aliquots of each QC were thawed unassisted at room temperature. When completely thawed, the aliquots were refrozen at -20 °C for at least 20 hours. This freeze-thaw cycle was repeated four more times. Data were measured in triplicates.

Statistical analysis

Statistical analyses were performed using Microsoft® Office Excel (2010) and the Statistical Package for Social Science 15.0. Curve fitting were achieved with Sigma Plot 12.

Results

Minimum required dilution

In this study, the acceptance criterium for MRD was based on percent of background signal reduction ≤ 100 % for at least 80% of the evaluated serum samples. The background signal reduction was upper 100% in all serum samples using dilution factors from 5 to 40 (Table 1).

In the dilution range from 1:80 to 1:160, most of the samples passed the acceptance criterium, which suggested that MRD could be in a dilution range near to 1:100. Percent of background signal reduction in the range from 1:85 to 1:100 (Table 2) were calculated by interpolation data from Table 1, using a logistic regression of five parameters.

As MRD was chosen 1:100, since in 90% of studied samples the background signal reduction was less than 100%.

Recovery

To determine recovery in this ELISA, dilution curves of anti-EGF antibodies was achieved by serial dilutions of positive control, using two types of diluents: Sample matrices at MRD or the DB. Spiked samples (theoretical spiking concentrations of 0.94, 3.8 and 15 ng/mL) had overall recoveries varying from 81.67 to 115.36% in matrix from healthy donors, and from 82.08 to 106.03% in matrix from patients treated with Heberprot-P® (Figure 1).

Screening cut-point

Data distribution was evaluated from each run,

Table 1: Percent of background signal reduction for ten serum samples (M1 to M10) from healthy donors.

Dilution factor	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
5	326.2	183.8	589.2	471.5	1275.4	1255.4	470.0	373.1	290.0	250.8
10	250.8	183.8	463.8	372.3	904.6	1009.2	280.8	286.9	222.3	228.5
20	171.5	155.4	328.5	220.8	490.8	795.4	190.8	156.9	138.5	138.5
40	106.2	87.7	200.8	115.4	169.2	636.9	123.8	123.1	108.5	96.9
80	80.0	46.2	113.1	66.2	101.5	414.6	60.8	53.1	95.4	53.1
160	48.5	34.6	56.9	46.9	74.6	365.4	40.8	38.5	46.9	40.0
320	30.0	29.2	41.5	16.2	28.5	276.2	16.9	32.3	25.4	17.7
640	16.2	18.5	17.7	11.5	20.0	160.8	14.6	22.3	21.5	16.9

Table 2: Percent of background signal reduction determined by interpolation of dilution range from 1:85 to 1:100, using a logistic regression of five parameters.

Dilution factor	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
85	71.8	47.2	107.0	63.1	79.7	453.1	65.7	61.6	73.2	54.8
90	69.3	45.4	101.7	60.1	75.3	442.5	63.0	59.4	70.9	52.8
95	67.0	43.8	100.9	57.5	71.6	432.7	60.5	57.4	68.8	50.9
100	64.9	42.4	92.5	55.0	68.4	423.5	58.2	55.6	66.9	49.2

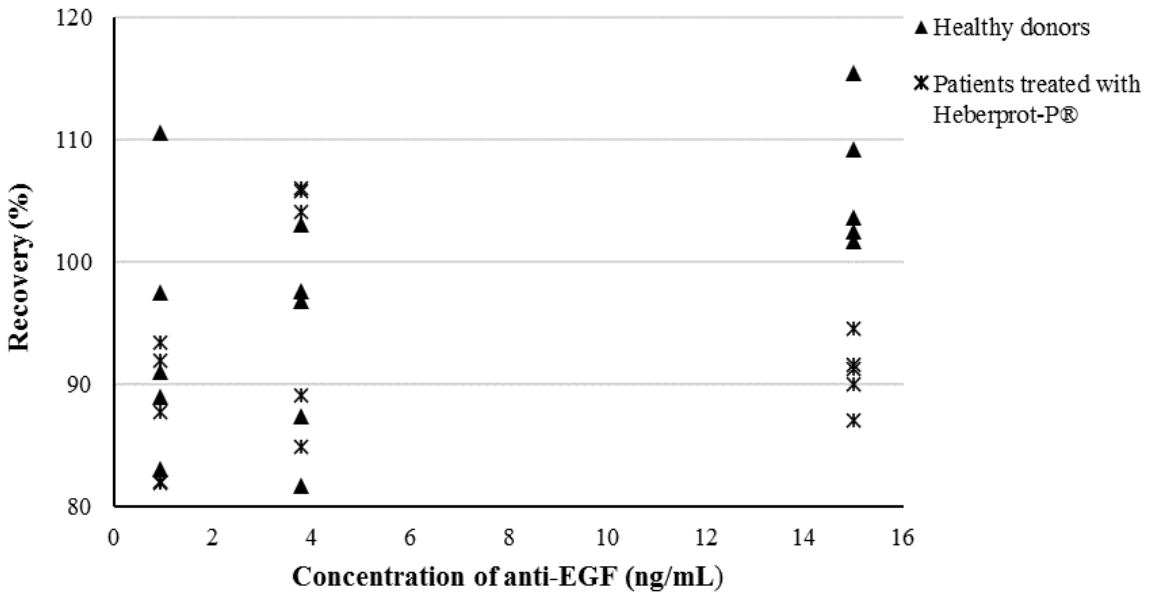

Figure 1: Results of the analysis of recovery in matrices from healthy donors and from patients treated with Heberprot-P®, spiked with known amounts of antibodies anti-EGF. Theoretical spiking concentrations: 0.94, 3.8 and 15 ng/mL.

after excluding the outliers. Shapiro-Wilk test verified the normality of ln-transformed data ($p \geq 0.05$), and it supported the use of parametric method (mean ($\ln A_{405nm}$) + 1.645 × SD) to define the screening cut-point.

The estimation of SD was assessed by a variance component analysis. The restricted maximum likelihood method within the framework of a nested ANOVA considered the following order of factors:

Analyst, day, plate, sample subgroup and sample. The "sample" factor was responsible of the 70.58% of total variance of the study (Table 3); it means that biological variability was determinant in the assay results.

The parametric cut point and the mean of negative control, both at natural logarithmic scale, were -2.5820 and -3.1701, respectively. The normalization factor, calculated by using the difference of both numbers, was 1.80.

Means were significantly different ($p \geq 0.000$) between repeated runs and variances were homogenous (Levene's test, $p = 0.355$). These results sustained the use of floating cut point for the screening, by the formula:

CP = mean A_{405nm} (Negative Control in clinical assay) × 1.80

Confirmatory cut point

Serum samples from 60 healthy donors, distributed like screening cut point, were incubated with an excess of antigen to preoccupy antibody binding sites. By using this incubation step, it is possible to distinguish between true positive samples (absence of signal) and false positive samples (presence of signal).

The percentile 1 of variable R was 0.1341. The confirmatory cut point (99% confidence) calculated by the formula $CP_{0.99}$ = 100 - (R × 100) was 86.59%.

Sensitivity

Serial dilutions of the positive control, spanning the assay cut-point in six assay runs by two operators, revealed a mean concentration at assay cut-point of 158.8 ng/mL of serum, with a SD of 27.5 ng/mL. Consequently, the sensitivity (aiming at a 5% failure rate) was calculated to be 158.8 ng/mL + (2.015 × 27.5 ng/mL) = 214.2 ng/mL.

Precision

Intra-assay precision for the screening ELISA was evaluated by the variation of the measurement of lnA_{405nm} in three concentration points of positive control and in negative control. Intra-assay CV of the screening ELISA was lower than 6.81% while inter-assay CV ranged from 7.63 to 13.70% (Table 4).

In the confirmatory ELISA, the inter-assay precision was assessed by the variation of the percent of inhibition in three concentration levels of positive control. The intra- and inter-assays CVs of the confirmatory ELISA did not exceed the 15.32% (Table 5).

Specificity

The determination of the percent of inhibition took into account the background signal reduction, and it was close to 100% in all cases (Table 6).

Quality controls confidence intervals

The confidence intervals (99%) for each QC were summarized in Table 7.

Table 3: Estimation of standard deviation of ln-transformed A_{405nm} by restricted maximum likelihood method within the framework of a nested ANOVA.

Variance components	Estimation	% Variability
Analyst	0.0000	0.00
Day	0.0314	11.01
Plate	0.0037	1.30
Subgroup	0.0000	0.00
Sample	0.2013	70.58
Error	0.0487	17.08
Total Variance	0.2852	
Standard Deviation	0.5340	

%Variability = (Estimation of variance component/Total variance) × 100

Table 4: Intra- and inter-assays precision in the screening ELISA.

			CV (%)	
Control	**Variance Error**	**∑ (Variance Components)**	**Intra-assay**	**Inter-assays**
Negative	0.0046	0.0058	6.81	7.63
High Positive (0.938 ng/mL)	0.0039	0.0141	6.28	11.91
Medium Positive (3.75 ng/mL)	0.0013	0.0186	3.60	13.70
Low Positive (30 ng/mL)	0.0017	0.0090	4.06	9.50

CV (%): Coefficient of variation expressed as percentage.

Table 5: Intra- and inter-assays precision in the confirmatory ELISA.

				CV (%)	
Concentration of Positive Control	**Mean % Inhibition**	**Variance Error**	**∑ (Variance Components)**	**Intra-assay**	**Inter-assays**
0.938 ng/mL	61.91	8.2448	41.502	10.41	13.32
3.75 ng/mL	71.81	11.004	23.543	6.78	15.32
30 ng/m)	96.39	0.1550	0.9930	0.41	1.03

CV (%): Coefficient of variation expressed as percentage.

Table 6: Estimation of specificity. Values represent the mean ± standard deviation of data from three replications.

Positive Control concentration (ng/mL)	**A_{405nm} inhibited**	**A_{405nm} non-inhibited**	**Inhibition (%)**
30.0	-0.019 ± 0.006	1.263 ± 0.308	101.46
7.50	-0.007 ± 0.002	0.392 ± 0.158	101.79
1.88	-0.002 ± 0.001	0.081 ± 0.049	102.47

Table 7: Determination of the confidence intervals for quality controls.

Quality Control	**Equation for the confidence interval**	**Mean**	**t (0.01, DF = 5)**	**SD**	**Confidence interval (99%)**
NC	mean (A_{405nm}) + t (0.01, 5df) × SD	0.063	3.365	0.0104	$A_{405nm} \leq 0.098$
LQC	mean (LQC/NC) ± t (0.01, 5df) × SD	3.980	4.032	0.4214	$2.28 \leq LQC/NC \leq 5.68$
HQC	mean (HQC/NC) ± t (0.01, 5df) × SD	13.80	4.032	1.6531	$7.13 \leq HQC/NC \leq 20.5$

NC: Negative control; LQC: Low positive quality control; HQC: High positive quality control.

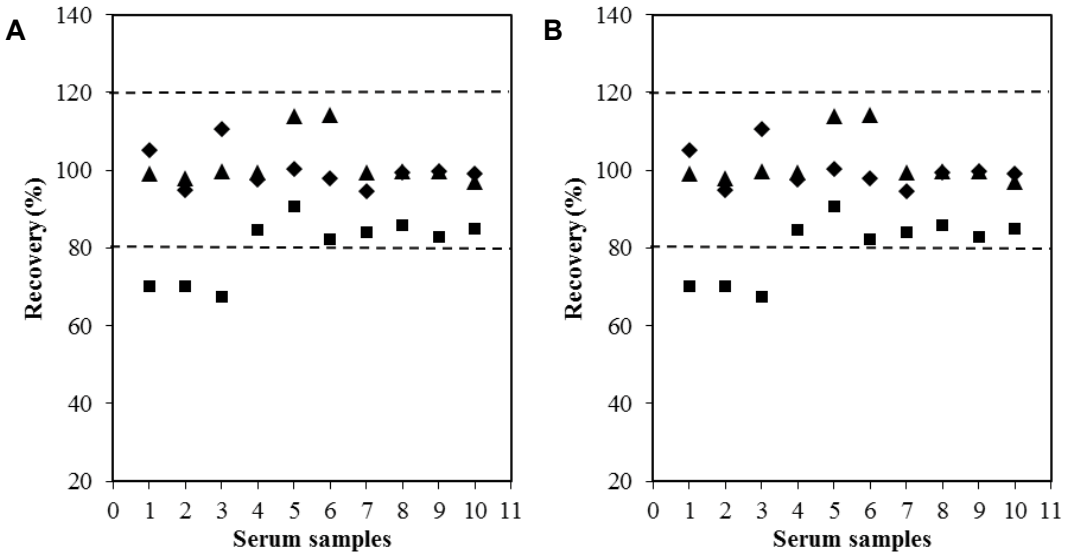

Figure 2: Recovery analysis for selectivity evaluation of serum samples with hemolysis (♦), lipemia (■) and from diabetic patients with foot ulcer (▲), spiked with low (A) and high (B) concentrations of anti-EGF antibodies.

Selectivity/Interference

Selectivity was assessed using serum samples with hemolysis, lipaemia and from diabetic patients with foot ulcer (target population). Samples were spiked with low and high concentrations of positive control, in order to calculate the recovery of the

samples in respect to the experimental controls. The percent of recovery, obtained with hemolytic samples and with samples from diabetic with foot ulcers, was in the acceptance interval at high and low concentrations of positive control (Figure 2).

However, the 50% of the lipemic samples spiked with low concentration of positive control and the 30% of the samples spiked with high concentration were not in the acceptance interval for recovery (Figure 2).

The interference was assessed by testing the effect of increasing concentrations of EGF on low concentration of positive control. A curve adjusted by a logistic regression of five parameters was used to fit these data in logarithmic scale, to interpolate the concentration of EGF corresponding to the response immediately below the cut point (Figure 3).

The mean of A_{405nm}NC was 0.074, and the cut-point for this ELISA was calculated by 1.80 × 0.074 = 0.133. It was taken 0.132 as A_{405nm} immediately below of cut-point for interpolation in the curve. The resulting tolerance limit of EGF was 27.3 ng/mL for low levels of anti-EGF antibodies (Figure 3).

Robustness

The robustness of this assay was verified by varying the incubation time in ± 5 min for antigen coating, samples and anti-human polyvalent AP-conjugated; decreasing of substrate concentration, and using of bovine serum albumin from three different suppliers. All these variations in the ELISA procedure did not alter the confidence intervals (99%) of QCs (Figure 4). But the variation of the PolySorp ELISA plate, from strip plate modules to compacted plate, affected the absorbance signal of negative control and the value of the ratio LQC/NC, which were outside the confidence intervals (99%) (Figure 4).

Stability of quality controls and samples

As suggested Mire-Sluis, et al. in 2004, the evaluation of stability was performed with samples con-

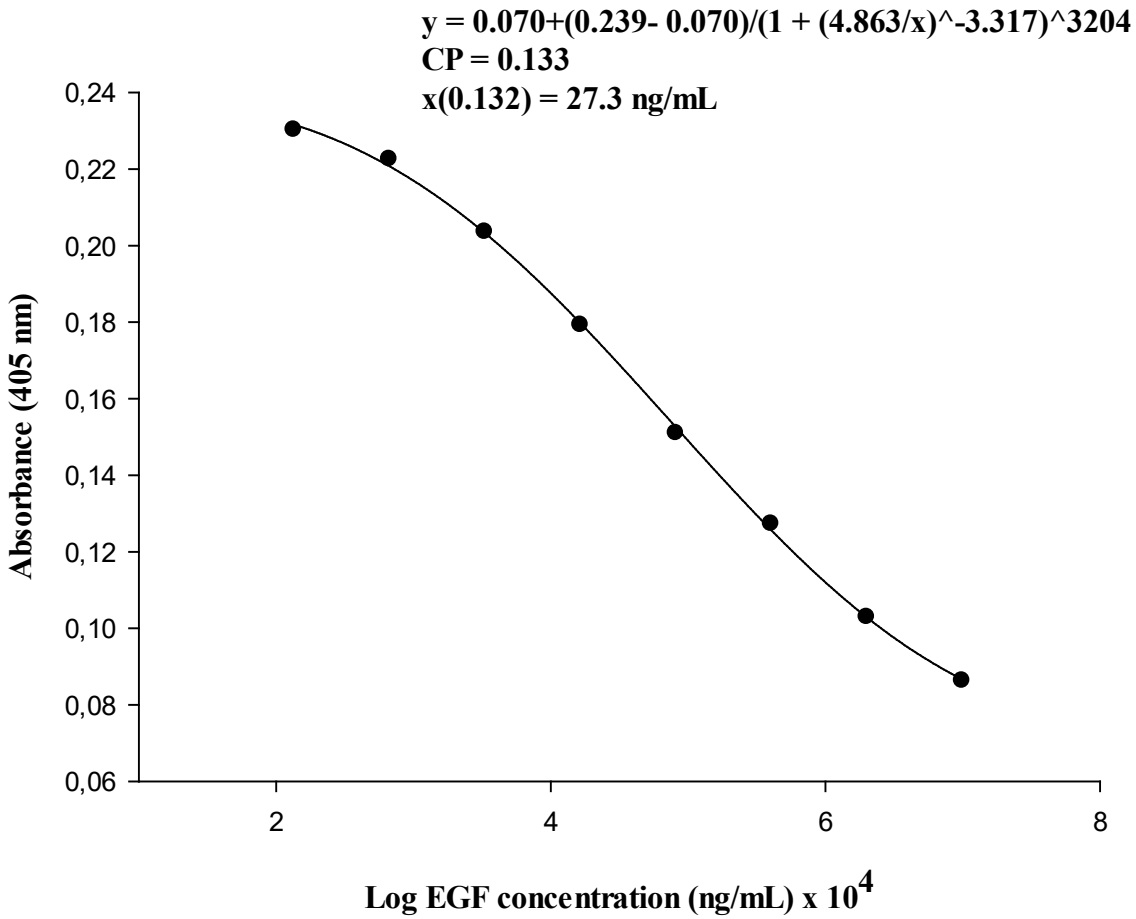

Figure 3: Calibration curve adjusted by a logistic regression of five parameters for the interpolation of the concentration of EGF immediately below the cut point (CP).

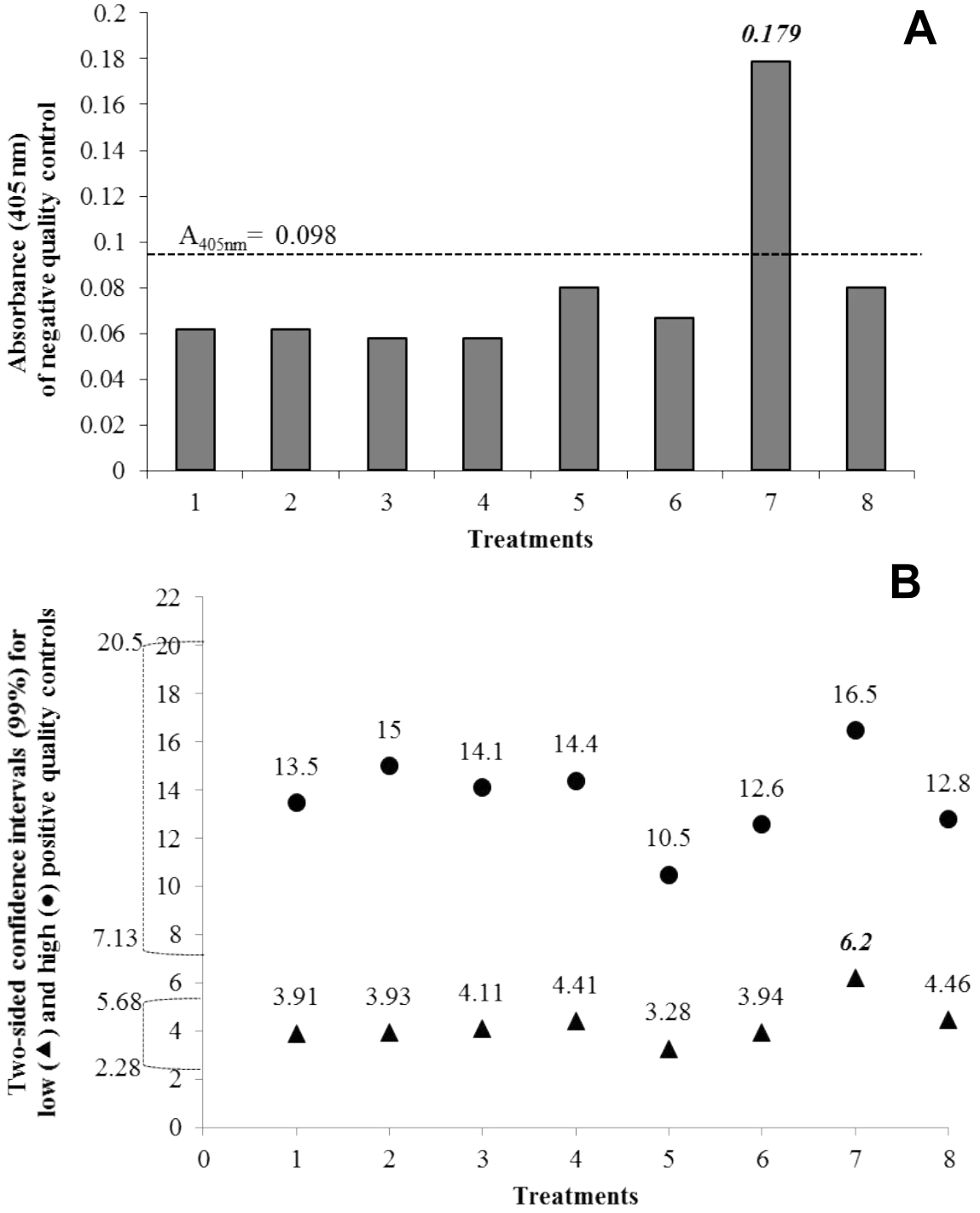

Figure 4: Evaluation of the robustness of the ELISA by the fulfilling of **(A)** Negative and **(B)** Positive quality controls confidence intervals. Values represent the mean of three replications. Treatments: **(1)** Variation in -5 min the plate incubation times for antigen coating, for quality controls and for anti-human polyvalent alkaline-phosphatase-conjugated; **(2)** Variation in +5 min the plate incubation times for antigen coating, for quality controls and for anti-human polyvalent alkaline-phosphatase-conjugated; **(3)** Decreasing in -0.2 mg/mL the substrate concentration; **(4)** BSA from Sigma; **(5)** BSA from Roche; **(6)** BSA from AppliChem; **(7)** Compacted PolySorp plate; **(8)** PolySorp strip plate module.

Confidence intervals (99%) of quality controls: A_{405nm}NC ≤ 0.098; 2.28 ≤ LQC/NC ≤ 5.68; 7.13 ≤ HQC/NC ≤ 20.5

taining high and low levels of anti-EGF antibodies. In this case, the HPC and LQC were used since QCs and samples are of the same species.

For the evaluation of the stability, QCs were submitted to temperatures used during processing and storage of samples during the ELISA, and to

Table 8: Results of the evaluation of the stability of quality controls.

Quality control measures	Short-term stability			Freeze–thaw cycles				
	3 h (22-25) °C	6 h (22- 25) °C	6 days (2-8) °C	1	2	3	4	5
A_{405nm} NC	0.058	0.057	0.057	0.054	0.056	0.055	0.057	0.057
CPA/NC	16.7	16.0	18.0	18.8	17.4	18.4	17.7	17.8
CPB/NC	4.12	4.17	4.29	4.42	4.17	3.92	4.04	4.02

Confidence intervals (99%) of quality controls: A405nmNC ≤ 0.098; 2.28 ≤ LQC/NC ≤ 5.68; 7.13 ≤ HQC/NC ≤ 20.5; Values represent the means of three replications.

the impact of freezing/thawing cycles. The results of stability assessments confirmed that QCs were stable in all assayed conditions (Table 8) and it was a validation of the stability of samples.

Discussion

We have validated a method for the detection of binding ADA against rhEGF, the active ingredient of Heberprot-P®, in human serum. The validation was undertaken following the recommendation of the principal regulatory agencies and international guidelines for the evaluation of immunogenicity of therapeutic proteins [2,3]. As recommended, the method included a two-tiered approach: A screening assay for identification of positive samples and a confirmatory assay to for ratify the specificity of binding antibodies. An ELISA format was used for both tiers.

Patient serum samples should be diluted to maintain a reasonable ability to detect ADA. Ideally, the MRD is the smallest dilution necessary for the detection of ADA in biological matrix with least interference, by reducing the signal to noise ratio [2]. The MRD for serum samples used in the present assay was 1:100, which was in agreement with recommendations for bioanalytical methods validation [3]. Higher dilution may outcome in false detection of a negative response when patients have low levels of ADA.

A spike-and-recovery experiment was designed to confirm if the MRD was adequate [7] and as accuracy criterion of the immunoassay. The recovery test was performed by using three anti-rhEGF concentrations [9,10]. The percent of recovery was in the acceptance range, and it validated the accuracy of the method and suggested a greater confidence of ELISA compatibility with the proposed sample, because significant differences between the sample matrix and the DB did not exist. The MRD was appropriate since all samples were diluted 1:100 and in all of them the recovery fulfilled the pre-established acceptance criterion.

The ELISA satisfied the specifications for precision in accordance with the regulatory guidelines [2,3]. Precision is the closeness of agreement between independent test results obtained under stipulated conditions. Thus, validating assay precision is important to the assessment of ADA, because assay variability is the basis for defining the cut points and ensuring that low positive samples are detected as positive.

The specificity of the assay was confirmed since the antigen was able to significantly inhibit the response of the positive control at high, medium and low concentrations, suggesting that the inhibition occurred independently of the quantity of target analyte, anti-rhEGF antibodies, present in the sample [11]. The binding reagent was specific such that no cross-reactivity occurred with structurally related compounds present in the matrix or with other assay components.

The assay remained unaffected by small changes in critical method parameters, but it was not robust to the variation in the format of the PolySorp plate, confirming the particular susceptibility of bioassays to variations in the procedure. Samples and QCs were stable at the most used temperature conditions, supporting that sample handling before, during and after analysis do not influence the assay results.

The screening assay should be sensitive enough for detecting all clinically relevant antibodies (including IgM and IgG subclasses) in all ADA positive patients. The use of an anti-human polyvalent alkaline-phosphatase conjugated aimed at the detection of all antibodies classes. The high sensitivity of this screening assay is paramount, thus, low false

positive rate is tolerable, even desirable (preferably 5%) but false negative results are unacceptable. Recently, FDA recommends that screening and confirmatory IgG and IgM ADA assays achieve a sensitivity of at least 100 ng/mL, although a limit of sensitivity greater than 100 ng/mL may be acceptable depending on risk and prior knowledge [1]. In the present work, sensitivity was calculated to be 214.2 ng/mL. Even when this figure is above the 100 ng/mL recommended by FDA, we considered it as adequate. The relative long history of thousand patients treated with Heberprot-P® with pertinent efficacy and without important adverse events [12,13], may suggest a low immunogenicity of this therapeutic product or that higher concentrations of antibody are needed to develop an antibody-mediated adverse event. This is a speculative argument since the proper measure of antibody concentration and its correlation with therapeutic responses or adverse event in clinical setting have not been done. Furthermore, it is widely known that the comparison of antibody concentration against different therapeutic products may be spurious due to the nature of the molecule being measured: A complex mixture of antibodies with different affinities. In this regards, the source of positive control is vital. The assessment of assay sensitivity relies almost exclusively on the characteristics of the positive control [7]. In our assays, the positive control was a pool of human sera from patients hyper immunized with rhEGF. The nature of this positive control deserves further discussion since it is different from the majority of positive controls reported in the literature. The obvious sources of positive controls are sera from immunized animals [14,15]. We have taken advantage from the fact that the active ingredient of Heberprot-P®, rhEGF, is the same of CimaVax-EGF vaccine, which is approved and widely used in Cuba for the adjuvant treatment of lung cancer [6]. This constitutes an accessible supply of human serum with high titers of anti-rhEGF antibodies. The fact that the antibodies in both clinical samples and positive control came from the same species (human) allow the use of the same conjugate in our ELISA, and could make the signals obtained from clinical samples more comparable to those signals obtained from the positive control.

Common nonspecific interferences in serum samples are due to hemolysis and lipemia [16]. This study demonstrated that hemolytic samples and serum samples from blank population did not affect the ability of the ELISA to detect ADA, but lipemic sera interfered in the assay, since they caused a deviation of the measured value from the true value. The most important substance to test for interference is the drug itself: rhEGF. It is expected that samples containing drug will exhibit interference due to competition for product specific antibodies between endogenous EGF and that used in the assay system [7]. The drug tolerance of the assay, like sensitivity, is highly dependent upon the positive control used to characterize it [8], and in this case it was human serum. Thus, the concentration of rhEGF corresponding to the response immediately below the cut point is the drug tolerance limit of the assay, the lowest concentration of drug that prevents the detection of the low positive control signal [8]. Our results confirmed that the ELISA is tolerant to endogenous concentration of EGF higher than the normal levels in serum for healthy males and females, 0.78 and 0.60 ng/ml, respectively [17]. The interference of exogenous rhEGF from Heberprot-P is irrelevant, since it is cleared as early as an hour post-administration and no accumulation after repeated injections [18].

Since the first demonstration that intralesional administration of recombinant human epidermal growth factor promotes granulation and healing in advanced DFU [4], Heberprot-P® was approved as adjuvant treatment of DFU. A recent meta-analysis concluded that rhEGF has the highest probability of being the best growth factor for the treatment of DFU, and it was significantly associated with a higher proportion of complete healing in conjunction with standard of care [5]. Those evidences suggest that the safety profile of Heberprot-P® is favorable and the probability of serious and frequent adverse events due to the presence of anti-rhEGF antibodies is low, however, this fact has not been properly evaluated. As the immunoassays described in this work have satisfied the basic characteristics required by regulatory agencies, we concluded that they could be reliable tools for the evaluation of immunogenicity of Heberprot-P® in well-designed clinical studies.

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