Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 403–408

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Novel and sensitive ELISA for the rapid quantification of recombinant p64K protein

Alberto Leyva[a,*], Julio C. Sánchez[a], Denis Álvarez[b], Bárbara Pérez[a], Lissette López[a], Shaily Paz[c], Edel Torres[c], Tatiana González[a], Milagros Font[a], Neyda Hernández[a], Rodolfo Valdés[b]

[a] Process Control Department, Center for Genetic Engineering and Biotechnology, Ave 31 between 158 and 190, P.O. Box 6162, Havana 10600, Cuba
[b] Production Department, Center for Genetic Engineering and Biotechnology, Ave 31 between 158 and 190, P.O. Box 6162, Havana 10600, Cuba
[c] Production Department, Center for Genetic Engineering and Biotechnology, Circunvalante Norte, S/N, Apdo. Postal 83, Código Postal 60 200, Sancti Spiritus, Cuba

ARTICLE INFO

Article history:
Received 11 November 2010
Received in revised form 27 January 2011
Accepted 31 January 2011
Available online 3 March 2011

Keywords:
ELISA
Validation
p64k-r
Manufacturing process
Conjugated vaccine

ABSTRACT

The antigenic P64k protein from the pathogenic bacterium *Neisseria meningitidis* has been used as an immunological carrier in several conjugated vaccines. The aim of this report was to develop and validate a sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for the quantification of recombinant p64k protein, to perform both manufacturing process and identification in different vaccine preparations. Validation studies were performed according to the guidelines of the International Conference of Harmonization (ICH). The reference curve showed to be precise and accurate over the entire linear range of 1.25 and 20 ng/mL with a limit of quantification validated to 1.25 ng/mL. The intra- and inter-assay coefficient of variation ranged from 0.35 to 6.65% and 4.70 to 10.63%, respectively. The ANOVA test used in the specificity/interference study revealed parallelism among curves ($p > 0.1$), which indicates the lack of interference in the working range. Recovery obtained from the accuracy test, using three concentration levels, varied between 94 and 111%, confirming the assay's reliability. The short-term study shown the P64k is stable to −20 °C up to 1-week. This ELISA was fully used to assess its manufacturing process and molecular interaction issues in several vaccine preparations. Thus, this immunoassay could be an excellent analytical choice to characterize the quality of that recombinant protein in several contexts as manufacturing process and molecular conjugates.

1. Introduction

Protein p64k from *Neisseria meningitidis*, has been described as an outer-membrane dihydrolipoamide dehydrogenase [1,2]. It has been recognized by hyper immune rabbit serum, also by sera from the convalescent Cuban and Norwegian patients, which indicates that this protein is highly immunogenic [3,4].

P64k was cloned and expressed in *Escherichia coli* [1] with the objective of designing a broad spectrum recombinant subunit vaccine. Besides, is a very attractive fusion and carrier protein due to the high levels of expression that can be achieved [5] and its highly immunogenic properties, proven in several vaccine preparations against viral and bacterial diseases [6–8]. In addition, the active induction of EGF specific antibodies is an emerging concept in cancer therapy with encouraging results [9]. Consequently, recombinant p64k (p64k-r) has been also used as a novel cancer vaccine developing a self-reactive antibody response against human Epidermal Growth Factor (EGF), in patients with histological proven malignant carcinomas [10].

An extensive characterization of this protein has been carried out e.g. the three-dimensional structure of this protein has been elucidated [11]; several polyclonal and monoclonal antibodies against this recombinant protein have been obtained, and used to characterize this antigen [12,13].

However, the production at large scale of this protein, process and quality control has been not reported yet. ELISAs for detection of recombinant protein have been reported [14]. Nevertheless, a sandwich ELISA can be difficult to develop and validate due to the signal amplification of both specific and non-specific components. Our current researches efforts are aimed at develop and validate a novel sandwich ELISA for the quantification of recombinant p64k protein, used as immunological carrier in vaccine preparation. To this end, analytical technique used to tightly control biopharmaceutical manufacturing processes, play an important role for the characterization of critical product/process attributes of final product characteristics. In this case, enzyme-linked immunosorbent assay (ELISA) has been considered extremely useful for the detection of recombinant protein [15], because it is simple, offers a

* Corresponding author. Tel.: +53 7271 6022x5113; fax: +53 7271 4764.
E-mail address: alberto.leyva@cigb.edu.cu (A. Leyva).

0731-7085/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2011.01.041

suitable sensitivity, and is useful in providing quantitative results. Also, would be a valuable tool for characterizing the final product and monitoring the batch-to-batch consistency.

Validation of an analytical procedure confirms that the method is suitable for the intended purpose [16], providing documental evidence that the assay will consistently meet its pre-determined specifications and quality control attributes [17,18]. For this reason, validation and process control are important in spite of problems that may be encountered [19].

In the present paper, we describe the generation of polyclonal antibodies (PAbs) for p64k-r protein, and employment of this PAbs in development and validation of a highly sensitive sandwich ELISA, which has been developed and validated to quantify the p64k-r carrier during its manufacturing process. In that sense, the dual-antibody sandwich ELISA (DAS ELISA) is one of the most sensitive and specific techniques for quantifying molecules in solution. This method has not been reported elsewhere before for this protein. We show that ELISA method is a valuable tool for characterizing the p64k process production and provide information on variation in the final product.

2. Material and methods

2.1. Production of recombinant p64k protein

The p64k-r protein was manufactured at the Center for Genetic Engineering and Biotechnology of Havana. Under carefully controlled multiplication conditions, the recombinant *E. coli* strain, which contains the entire *lpdA* gene encoding for the p64k-r protein under control of the tryptophan promoter [20], were passed from shake flasks into bioreactors. Some chromatography steps before obtaining the pure protein (≥98%) were carried out.

2.2. Buffer, manufacturing process samples and vaccine preparation

All buffers were made using injection and purified grade waters. The following buffers were used:

Resuspended (R) and Ion-exchange chromatography (Q) (20 mM tris, 6 mM EDTA, pH 7.2) (20 mM tris, 6 mM EDTA, 0.3 M NaCl, pH 7.2), respectively. Hydrophobic chromatography (B)(20 mM tris, 6 mM EDTA, 0.15 M NaCl, pH 7.2) were supplemented with concentrated $(NH_4)_2SO_4$. Gel filtration, Sephadex chromatography (G-25) and Active Pharmaceutical Ingredient (API) contain the same phosphate buffer. Manufacturing process samples used in the validation study match with the above described buffers. The four fused vaccine p64k-dengue (1–4) were provided by Vaccine Division at the Center for Genetic Engineering and Biotechnology of Havana, which correspond to antigenically distinct serotypes of dengue virus. Besides, two synthetic peptides comprising aa regions in the NS4 protein (aa 1689–1735) and the hypervariable region I (HVR I, aa 384–414) in the Hepatitis C virus E2 protein were conjugated to the P64k protein (NS4/p64k and HVR/p64k respectively) and provided by Hepatitis C Department. The vaccine EGF/p64k composition has as active principle a chemical conjugate of human recombinant Epidermic Growth Factor (hrEGF) and recombinant protein P64k. This vaccine preparation was provided by the Center of Molecular Immunology at Havana. The both reference material (p64k-04-0405), and the API Lot number 9005 used as standard and control in the ELISA assay respectively, were prepared in the Stability and reference material Department at the Center for Genetic Engineering and Biotechnology of Havana.

2.3. Antibodies

A polyclonal antiserum to p64k was developed in rabbit at the Center for Genetic Engineering and Biotechnology, Sancti Spiritus. A female adult rabbit was immunized with 2.5 μg of purified p64k in completed Freund's adjuvant by subcutaneous injection. After 4 weeks, the rabbit received intramuscular booster injection of the 1:1 emulsion of p64k-r 2.5 μg and Freund's incomplete adjuvant. The final immunization was conducted by intravenous injection of 2.5 μg of the p64k-r. After 3 days, the rabbit was checked for the presence of the antibodies using direct ELISA and then the rabbit was bled. Purification of Polyclonal antibodies (PAbs) from the serum was carried out using a protein A affinity chromatography method described by Ey et al. [21]. The serum was loaded to the column and then it was washed with 100 mM phosphate buffer, pH 7.4 (300 cm h^{-1}). After that, bound IgG was eluated with 100 mM citrate buffer, pH 3 (100 cm h^{-1}). The eluted IgG was neutralized with 2 M Tris–HCL buffer (pH 9) and a buffer exchange was carried out by means of a gel filtration chromatography using Sephadex G-25 (Amersham-Biosciences, Uppsala, Sweden), in a BP113/120 column using as equilibrium buffer 20 mM Tris/150 mM NaCl, pH 7.6. The volume of the samples ranged from 1 to 3 L and the flow rate used was 129 cm h^{-1}. Finally, purified antibodies were stored at −20 °C. Monoclonal antibody (Mab) CBNt.p64k was raised against p64k protein as described by Nazábal et al. [12]. This Mab was conjugated to horseradish peroxidase (HRP) by the method described by Nakane and Kawoi [22].

2.4. Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed as follows: Polystyrene 96 well microtiter plates (Nunc-Immunoplate Maxisorp, Nunc, Denmark) were coated 20 min at 50 °C with 100 μL/well of a specific p64K.2 polyclonal antibody (10 μg/mL) in carbonate/bicarbonate buffer, pH 9.6. Plates were washed in phosphate buffer containing 0.05% Tween 20 (PBS-T). One hundred microliter of standard, control and samples were added in duplicate to the appropriate wells, and the plates were incubated during 30 min at 50 °C. Subsequently, wells were washed five times and incubated with other anti-p64k Mab (Nt.p64k-HRP) conjugate in 1:20,000 dilutions for 1 h at 37 °C. Finally, after another washing, 100 μL of enzyme substrate solution (*o*-phenylnediamine, 0.015% H_2O_2 in citrate buffer, pH 5.0) was added to each well and the plates were incubated for 10–20 min in the dark at 23 °C. The reaction was stopped by addition of 50 μL of 2 M H_2SO_4 and immediately read at 492 nm using a microELISA reader (Labsystem, Helsinki, Finland).

2.5. Linearity and working range

The linearity of the method was established by analyzing standard concentration in a wide range from 0.2 to 400 ng/mL. The least-squared method was applied for obtaining a function describing a linear model. Regression coefficient (r^2), *y*-intercept and slope were analyzed. Working range was established between the highest and lowest concentration values with satisfactory accuracy and precision (coefficient of variation (CV) < 10%).

2.6. Quantification and detection limit

The quantification limit (QL) was defined as the smallest concentration of p64k-r protein with an intra-and interday imprecision <20% [23]. We accepted the lowest value. The detection limit (DL) of the assay was calculated as follows:

DL = 3.3 SD [mean of zero standard]/Slope

2.7. Precision and accuracy

The intra- and inter-assay precision was determined by a repetitive analysis of the production samples ($n = 6$ for intra-plate and $n = 9$ for interplate analysis). For both precision assays the acceptance criteria were coefficient of variation [CV (%) = S.D/mean × 100%] lower than 10 and 20% respectively. Spiking recovery was determined by adding 10, 5 and 2.5 ng/mL to each of the diluted manufacturing process samples. Recovery was expressed as the bias of the percentage of error between the observed value and the true value.

2.8. Specificity

To investigate effects of the matrix in each manufacturing process sample on the p64k-r protein, a statistical comparison to slope and intercept using a variance analysis (ANOVA) between the reference curve and other curve spiked with each buffer of manufacturing process and protein impurities was performed. Additionally, the quantification of positive control calculated by was compared by using the CV < 20% in each curve.

2.9. Determination of total protein concentrations to manufacturing process samples

Protein concentration was determined according to Bradford's method with slight modifications [24]. Briefly, 96-well microplate was applied as the reaction well and absorbance was measured by microplate reader at 620/450 nm (Labsystem, Helsinki, Finland). The amount of protein was calibrated against bovine serum albumin used as reference standard in the range between 5 and 40 μg/mL. Several manufacturing process samples of p64k-r were measured in the same microplate and results were reliable (the determination coefficient of the calibration curve was above 0.99).

2.10. SDS–PAGE electrophoresis

Samples were separated in a SDS–PAGE gel (12.5%) as described by Laemmli [25]. Ten microgram of protein were applied in each well.

2.11. Statistical analysis

All statistical analysis was performed using Microsoft® Office Excel (2007) and the STATGRAPHICS Centurion XV.II professional version 5.0 programs (1994–2000).

3. Results and discussion

3.1. Assay development and optimization

The assay was based on the capture of the p64k protein by specific PAbs anti-p64k.2 and, subsequently formation of immune complex by the reaction of the MAb CBNt.p64k-HRP. Assay optimization involved the study of several parameters, including the concentration of antibodies, sample dilution, incubation time and choice of primary and secondary antibodies and working buffer. The optimal concentration for capture, primary antibodies binding and conjugate addition were 10 μg/mL and 1:20,000, respectively. It was established that when compared to the other working buffers, the phosphate buffer/0.1% Tween 20 gave the highest OD492 nm ratio between positive and negative samples. Finally, during microplate coating, temperature and incubation time (1 h at 37 °C, 16 h at 4 °C, or 20 min at 50 °C) showed similar results (Data not shown). This allowed to use one condition or another indistinctively. Standardization of this ELISA revealed that the combination of the PAbs anti-p64k.2 and the Mab Nt-CB.p64k as the capture and detection antibodies, respectively, resulted in the best signal-to-noise ratio. In comparison with other antibodies tested (data not shown), this combination gives a minimal background, highest specificity and accurate in the quantification of p64k-r protein.

Table 1
Acceptance criteria and mean characteristics of sandwich ELISA anti-p64k-r.

Assay parameter	Results	Acceptance criteria
Repeatability	0.35–6.65%	%CV < 10
Intermediate precision		%CV < 20
Analysts		
1	0.85–6.65%	
2	0.46–3.22%	
3	0.35–1.89%	
Inter analysts	4.70–10.63%	
Accuracy	93.2–104.5%	80–120%
Limit of quantification	1.25 ng/mL	
Limit of detection	0.45 ng/mL	
Linearity	Working range: 20–1.25 ng/mL $r^2 > 0.98$ p-value < 0.01	

3.2. Assay validation

3.2.1. Linearity and working range

Nowadays, validation method is a process demonstrating that an analytical procedure will successfully meet or exceed the minimum standard recommended by FDA [17] and ICH guidance [15,18] for accuracy, precision, selectivity, sensitivity, and linearity (Table 1).

Linear regression curves were determined by plotting the concentration of reference material of the p64k-r protein versus the absorbance on the linear scale. Variance analysis test demonstrated that the calculated *F Snedecor* associated probability was less than 0.01, indicating a relatively strong relationship between the variables in this range, and the variance analysis of Lack-of-fit shown a p-value of 0.4456 confirming that the model appears to be adequate for the observed data at the 95.0% confidence level. The determination coefficient was >0.99 in all cases; which indicates that the model as fitted explains 99.80% of the variability between both variables. In this assay, the quantification limit was 1.25 ng/mL determined with precision and accuracy; CV = 5.6% and 98% recovery, respectively. Detection limits (DL) of this method were calculated by the interpolation on the standard curve, as the analyte concentrations corresponding to the mean signal of blank three times its standard deviation. The ELISA assay has a DL of 0.45 ng/mL. This value is at least 60-fold more sensitive than the reported for Tetanic Toxoid immunological carrier [26]. Under fixed working conditions, the assay was linear between 1.25 and 20 ng/mL, demonstrating the higher sensitivity of this assay in respect to other reported ELISA [27], while the mean recovery was 99%, which is in agreement with FDA recommendations for bioanalytical methods [17]. Regression equation from seven different standard curves was defined as:

$$y = 0.05432 + 0.04758 \times x$$

3.2.2. Matrix interference and assay sensitivity

Matrix effect defined as "the sum of the effects of the entire component with the exception of the analyte" [28] was determined in the specificity test. In our case, the dilution buffer was sufficient to avoid none specific signal in the immunoassay test. The comparison of these spiked curves did not show significant differences ($p_{\text{intercepts}} = 0.5189$; $p_{\text{slope}} = 0.1517$, $\alpha = 0.05$) indicating the lack of interference in the working range. The positive con-

Table 2
Results of accuracy assessment.

Assays	Concentration applied p64K (ng/mL)	True amount recovery (ng/mL)/%
1	10	9.8/98
2	10	10.5/105
3	10	11.1/111
Mean	–	10.5/–
SD	–	0.4
CV%	–	1.03
1	5	4.9/98
2	5	4.7/94
3	5	5.2/104
Mean	–	4.9/–
SD	–	0.9
CV%	–	2.15
1	2.5	2.6/104
2	2.5	2.5/100
3	2.5	2.5/100
Mean	–	2.5/–
SD	–	1.3
CV%	–	4.31

trol quantification showed a coefficient of variation lower than 10% between compared curves (data not shown). Specificity assays demonstrated that the ELISA detected only recombinant p64k protein without buffer interferences in the working range used and under the working condition fixed.

3.2.3. Accuracy

The most useful method to calculate accuracy is the recovery test. Moreover, the ICH guideline recommends the demonstration of accuracy over the whole working range. Spiked production process samples (final theoretical spiking concentrations of 2.5, 5 and 10 ng/mL) had overall recoveries varying from 94 to 111%, with a mean recovery of 100.8% (Table 2), which is in agreement with FDA recommendations for bioanalytical methods [17]. The statistical analysis of the hypothesis test concerning the mean value of a normal distribution did not reject the null hypothesis at 95% confidence level (p-values = 0.1668). Therefore, there are no meaningful differences between the expected and the real recovery values.

3.2.4. Precision

Precision is the agreement within a series of individual measurements of an analyte when the analytical procedure is repeatedly applied to multiple aliquots of a single homogeneous volume of a biological matrix [23]. For our experimental conditions the repeatability and intermediate precision percent ranged 0.35–6.65% and 4.70–10.63%, respectively indicating a low intra- and inter-assay variation and sample stability (Table 3). Therefore, this method fulfills with the specifications reported for analytical procedures validation [15,18].

3.3. Study of short-term stability with manufacturing process samples

A short-term stability study to the API was carried out. The stability of this sample was assayed up to 7 days post-production by incubating the preparation at −20 °C, 4 °C, room temperature (RT) and 37 °C. Significant differences between −20 °C and the rest of incubation temperatures was observed (>20%) variation between days from initial values (Fig. 1). This indicates that p64k-r protein is stable at least for 24 and 72 h at room temperature and 4 °C, respectability and for 1-week at −20 °C. This incubation time is sufficient to carry out the different vaccine preparation that contains the p64k immunological carrier.

Table 3
Precision for intra- and inter-assay CVs in different assays.

Samples	Precision CV %		
	Intra-assay	Inter-assay	Inter-analyst
Analyst 1			
R	2.3	2.3	6.5
Q	4.2	4.2	4.7
B	3.2	3.2	8.1
G	1.3	1.3	5.2
Analyst 2			
R	0.2	0.2	
Q	2.4	2.4	
B	0.9	0.9	
G	1.3	1.3	
Analyst 3			
R	2.3	2.3	
Q	0.9	0.9	
B	0.4	0.4	
G	3.4	3.4	

R, resuspended material; Q, ion exchange chromatography; B, hydrophobic chromatography; G, Gel filtration, Sephadex chromatography (G-25).

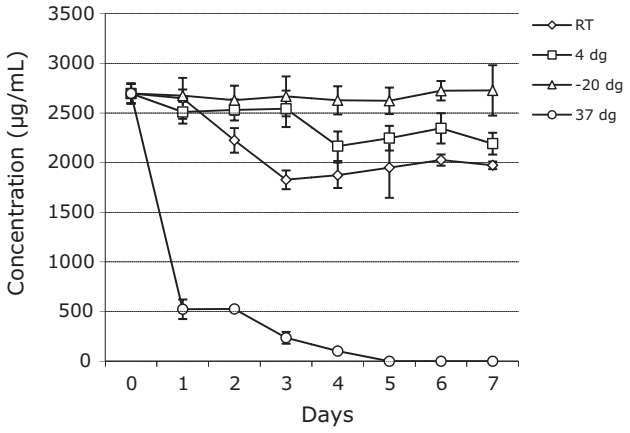

Fig. 1. Effect of incubation temperature on API p64k-r sample. Values correspond to the media of tree independent determinations.

3.4. Application of the developed ELISA to quantify recombinant p64k from both manufacturing process and conjugated vaccine samples

During large-scale production of recombinant proteins, its concentration is perhaps an essential monitoring parameter. Therefore, the primary application of this sandwich ELISA is to assess the manufacturing process consistency and provide information regarding

Table 4
Recovery of purification process step.

Process step	Volume (mL)	Protein (mg/mL)	ELISA (µg/mL)	p64k (g)	Recovery (%)	Purity (%)
R	25,000	0.376	137.59	3.40	100	72.68
Q	4000	0.777	544.16	2.18	22	89.45
B	3500	0.490	462.97	1.62	74	97.75
G	5200	0.285	281.94	1.46	90	98.54

R, resuspended material; Q, ion exchange chromatography; B, hydrophobic chromatography; G, Gel filtration, Sephadex chromatography (G-25).

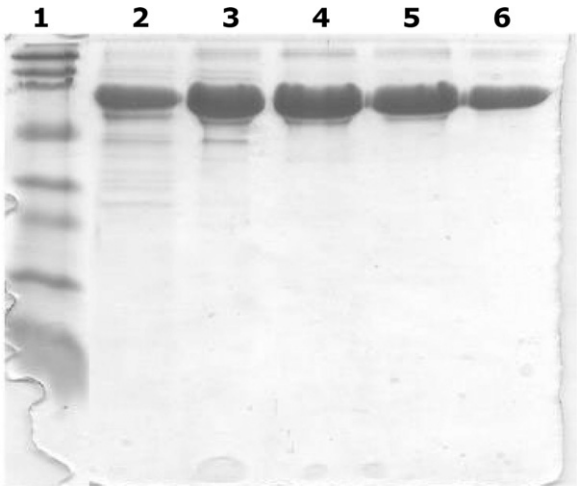

Fig. 2. The purity of p64k-r at different process stages was observed by SDS–PAGE. Ten microgram of each sample were loaded for analysis. Lane 1, Molecular weight marker; lane 2, resuspension sample; lane 3, ion exchange sample; lane 4, hydrophobic chromatography sample; lane 5, G-25; lane 6, API.

to batch-to-batch variation. In that sense, a validated assay is required by the current good manufacturing practice (cGMP) and for the assessment of the product during the manufacturing process [29]. The purification protocol and the process recovery are summarized in Table 4. An analysis of p64k-r of various purification steps is documented in Fig. 2 and the purity of the final p64k-r API sample was determined as >99% when analyzed by SDS–PAGE. The results showed that while each purification step was completed, an increasing of p64k-r protein recovery and purity was observed, which demonstrates the assay reliability and process consistency.

On the other hand, several vaccine preparations containing this carrier protein were evaluated. Firstly, different preparations with this protein, fused to envelope protein of dengue virus were detected in the ELISA assay. Surprisingly, the presence of this immunological carrier protein in EGF/p64k conjugated vaccine was not detected (Table 5). The true reasons for this unprecedented phenomenon are still unknown. However, this suggests that a chemical modification could take place in the carrier molecule during conjugation process and significantly affect recognizing sites of the antibodies and its corresponding epitopes. Similarly, the autologous molecule should be conjugated to these recognizing sites and totally or partially block such target places. Nevertheless, we believe that the implementation of this ELISA assay to evaluate the conjugating process would be a valuable approach as analytical control system.

Finally, statistical quality control for repeated assay runs during the quantification of p64k production process was performed using the control chart of the positive control. Fig. 3 shows a little variation (9.92%) between 25 individual runs where the common X-bar chart assumes variance

Table 5
Detection of p64k-r in different vaccine preparations.

Type vaccine	Nomenclature	Protein (mg/mL)	ELISA (μg/mL)	% detected
Fused vaccine	p64k-Dengue 1	1.60	542.98	34
	p64k-Dengue 2	0.75	176.09	23
	p64k-Dengue 3	1.00	182.90	18
	p64k-Dengue 4	1.40	110.29	8
Conjugated vaccine	NS4/p64k	1.90	286.25	15
	HVR/p64k	0.66	198.56	30
	EGF/p64k	1.00	0.060	0

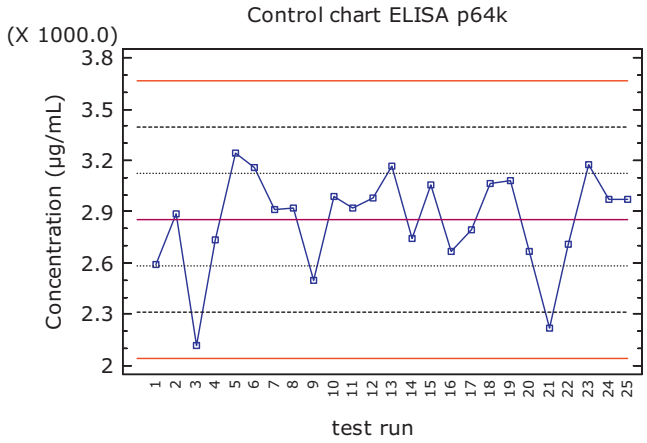

Fig. 3. Control chart of the positive control tested in the assay. Limits were established with 25 different values. Assays were carried out in a period of 3 months.

homogeneity and normal distribution for replicated measures per run, demonstrating the stability samples and assay consistency.

4. Conclusion

Summarizing, the new and sensitive ELISA developed in this paper could be used for the quantification of recombinant p64k. The validation results shown that this immunoassay is specific, accurate and precise in a concentration range between 1.25 and 20 ng/mL. Moreover, this ELISA can be successfully performed for both monitoring the recombinant p64k through manufacturing process and to characterize conjugated vaccines with this molecular carrier.

Acknowledgments

The authors wish to thank the Bacteria Department for providing the manufacturing process samples and the Process Control Department for the financial support of this study. We are also grateful to Jose L.Fdez.Sierra for reviewing the English.

References

[1] R. Silva, M. Selman, G. Guillen, L. Herrera, J.R. Fernández, L.I. Novoa, J. Morales, S. González, B. Tamargo, J. del Valle, et al., Nucleotide sequence coding for an outer membrane protein from *Neisseria meningitidis* and its use in vaccine preparations, Eur. Patent Appl. 0474313A2, U.S. Pat. 5 286484, 1992.
[2] R. Bringas, J.R. Fernandez, A lipoamide dehydrogenase from *Neisseria meningitidis* has a lipoyl domain, Protein. Struct. Funct. Genet. 21 (1995) 303–306.
[3] A. Musacchio, S. Gonzalez, E. Caballero, E.A. HØiby, E. Rosenquist, Antibody response against the 64 kDa protein from *Neisseria meningitidis* after systemic meningococcal disease, and after vaccination with two different serogroup B meningococcal vaccines among young adults in Iceland, in: J.S. Evans, S.E. Post, M.C.J. Maiden, I.M. Feavers (Eds.), Proceedings of the Ninth International Pathogenic Neisseria Conference, The Guidhall, Winchester, England, 1994, pp. 460.
[4] G. Guillen, A. Alvarez, R. Silva, V. Morera, S. Gonzalez, T. Carmenate, L.J. Gonzalez, R. Estrada, Y. Támbara, G. Padron, Expression in *Escherichia coli* of the lpdA gene; protein sequence analysis and immunological characterization of the P64k protein from *Neisseria meningitidis*, L. Herrera, Biotechnol. Appl. Biochem. 27 (1998) 189–196.
[5] R. Espinosa, E. Caballero, A. Musacchio, R. Silva, Production of a recombinant, immunogenic protein, P64k, of *Neisseria meningitidis* in *Escherichia coli* in fed-batch fermentes, Biotechnol. Lett. 24 (2002) 343–346.
[6] L. Alvarez-Lajonchere, G. Martínez, J. Morales, J.C. Aguilar, S. Dueñas, Immunization with HCV synthetic peptides conjugated to the P64k protein elicited strong antibody response in mice, Biotecnol. Apl. 20 (2003) 209–213 (Cuba's Journal).
[7] A.E. Pérez, F.O. Dickinson, F. Banderas, T. Serrano, R. Llanes, D. Guzmán, P. Díaz, A. Alvarez, M. Guirola, E. Caballero, L. Canaan-Haden, G. Guillén, Safety and preliminary immunogenicity ofMenC/P64k, a meningococcal serogroupC conjugate vaccine with a new recombinant carrier, FEMS, Immunol. Med. Microbiol. 46 (2006) 386–392.

[8] L. Hermida, R. Rodríguez, L. Lazo, R. Silva, A. Zulueta, G. Chinea, C. López, M.G. Guzmán, G. Guillén, A dengue-2 Envelope fragment inserted within the structure of the P64k meningococcal protein carrier enables a functional immune response against the virus in mice, J. Virol. Methods 115 (2004) 41–49.
[9] B. Garcia, E. Neninger, A. de la Torre, I. Leonard, R. Martinez, C. Viada, et al., Effective inhibition of the epidermal growth factor/epidermal growth factor receptor binding by anti-epidermal growth factor antibodies is related to better survival in advanced non-small-cell lung cancer patients treated with the epidermal growth factor cancer vaccine, Clin. Cancer Res. 14 (2008) 840–846.
[10] G. González, T. Crombet, M. Catalá, V. Mirabal, J.C. Hernandez, Y. Gonzalez, P. Marinillo, G. Guillen, A. Lage, A novel cancer vaccine composed of human-recombinant epidermal growth factor linked to a carrier protein: report of a pilot clinical trial, Ann. Oncol. 9 (1998) 1–5.
[11] I.L. de la Sierra, L. Pernot, T. Proge, P. Soludjian, M. Shiltz, R. Fourne, G. Padron, Molecular structure of the lipoamide dehydrogenase domain of a surface antigen from *Neisseria meningitidis*, J. Mol. Biol. 269 (1997) 129–141.
[12] C. Nazabal, T. Carmenate, S. Cruz, S. Gonzalez, R. Silva, A. Musacchio, M. Delgado, G. Chinea, Mapping of monoclonal antibodies specific to P64k: a common antigen of several isolates of *Neisseria meningitidis*, Can. J. Microbiol. 47 (2001) 158–164.
[13] S. Gonzalez, I. Viña, C. Nazabal, G. Cinea, E. Caballero, A. Musacchio, B-cell epitope mapping of the *Neisseria meningitidis* P64k protein using overlapping peptides, Biotechnol. Appl. Biochem. 32 (2000) 1–8.
[14] D. Geng, G. Shankar, A. Schantz, M. Rajadhyaksha, H. Davis, C. Wagner, Validation of immunoassays used to assess immunogenicity to therapeutic monoclonal antibodies, J. Pharm. Biomed. Anal. 39 (2005) 364–373.
[15] A. Leyva, J.C. Sánchez, R. Valdes, M. Font, L. López, N. Hernández, W. Ferro, Y. Medina, A combinatorial strategy of a new monoclonal ELISA and immunoaffinity chromatography using sodium deoxycholate to increase the recovery of multimeric proteins like r-HBsAg, Talanta 81 (2010) 314–319.
[16] ICH. International conference on harmonization of technical requirements for registration of pharmaceuticals for human use. Text on validation of analytical procedure: Text and methodology 2005, Q2 (R1), p. 1–13.
[17] U.S. Food and Drug Administration, Guidelines on General Principles of Process Validation, 1998, March.
[18] International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) Q2A: Validation of Analytical Methods (Definitions and Terminology) (October 1994).
[19] J.R. Sharp, The problems of process validation, Pharm. J. 1 (1986) 46–55.
[20] L.I. Novoa, J.A. Machado, J.R. Fernandez, J. Benitez, R.E. Narciandi, J.L. Rodríguez, M.P. Estrada, J. García, L.S. Herrera, Methods for the expression of heterologous proteins produced in fuse form in *E. coli*, use thereof, expression vector and recombinants strains. European. Patent. Application EP416673, 1991.
[21] P.L. Ey, S.J. Prowse, C.R. Jenkin, Isolation of pure IgG1, IgG2a, IgG2b, immunoglobulins from mouse serum using protein A-sepharose, Immunochemistry 15 (1978) 429–436.
[22] P.K Nakane, A. Kawoi, Peroxidase-labeled antibody a new method of conjugation, J. Histochem. Cytochem. 22 (1974) 1084–1091.
[23] V.P. Shap, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I. McGilveray, G. McKay, J.K. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Bioanalytical method validation—a revisit with a decade of progress, Pharm. Res. 17 (2000) 1551–1557.
[24] T. Zor, Z. Selinge, Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies, Anal. Biochem. 236 (1996) 302–308.
[25] U.K. Laemmli, Cleavage of structural proteins during the assembly of head of bacteriophage T4, Nature 227 (1970) 680–685.
[26] S. Šeatović, A. Inić-Kanada, M. Stojanović, I. Živković, R.M. Jankov, L. Dimitrijević, Development of sandwich enzyme-linked immunosorbent assay for determination of tetanus toxoid concentration, J. Immunol. Immunochem. 1 (2005) 31–44, http://www.informaworld.com/smpp/title~db=all~content=t713597271~tab=issueslist~branches=25-v2525.
[27] S. Sakamoto, W. Putalun, R. Tsuchihashi, S. Morimoto, J. Kinjo, H. Tanaka, Development of an enzyme-linked immunosorbent assay (ELISA) using highly-specific monoclonal antibodies against plumbagin, Anal. Chim. Acta. 607 (2008) 100–105.
[28] W.G. Wood, Matrix effects in immunoassays, Scand. J. Clin. Lab. Invest. Suppl. 205 (1991) 105–112.
[29] J. Lindholm, M. Johansson, T. Fornstedt, Guidelines for analytical method development and validation of biotechnological synthesis of drugs.Production of a hydroxyprogesterone as model, J. Chromatogr B. 791 (2003) 323–336.