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