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RABIES VIRUS IN McCOY CELL LINE - PART III (PURIFICATION)*

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ABSTRACT: During this work Sokol's classical method, slightly modified, was used in order to obtain the purified fraction of rabies virus.

A filtration in Sephadex G-50 column took place followed by another filtration in Sephadex G-75, eliminating the enzimatic treatment with DNase and RNase.

The elution in Sephadex G-50 separated two peaks with activity in 280nm (Absorbance). Peak I has separed a bovine albumine serum and Peak II has separated most of the viral fraction purified, free of such contaminats as serum or cellular proteins. Peak I was submitted to a saccharose gradient clumping all viral particles components and they were biochemically characterized throughout the electrophoretic separation in poliacrylamide gel SDS-PAGE and immunogenically throughout crossed immunoelectrophoresis.

Crossed immunoelectrophoresis was used to follow all the purification process, allowing the observation of which fractions were eliminated in each step of the process.

On the other hand, two advantages could be obtained when the bovine albumine serum was eliminated in second filtration through the Sephadex G-50 and when crossed immunoeletrophoresis technique allowed a complete peer-view of the process.

This aproach is very interesting when a large scale production process of purified antigen is involved for it allows the control of the purity degree and the attainment of a good quality control of the product.

DESCRIPTORS: Rabies virus, McCoy cell line, Purification, Crossed immunoeletrophoresis and Glycoproteins.

INTRODUCTION

According to Nogueira *(Part I and II)*[8,9] the evidence of cytopathic effect (CPE) in McCoy cell line infected with rabies virus is associated with virus replication and increase of the titer.

The rabies virus immunochemistry characteristics were observed during the process of purification.

Rabies virus multiplied and released in McCoy cell line, grown in suplemented media with foetal calf serum was purified according to Sokol's[11] classical method submitted to some modifications. It was used ERA strain of rabies virus obtained after several passages in McCoy cell cultures.

During the purification process it was used the crossed-immunoelectrophoresis technique according to Laurell [5] and Week[14], afterwards, this technique was standardized by Nogueira et alii[7], in order to identify the immunogenic fractions from anti-rabic vaccine Fuenzalida-Palacios[3].

The technique was adequate to make clear the purifiction process and constituted a simple and cheap method that could control the entire process.

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MATERIALS AND METHODS

VIRUS: Rabies virus. ERA strain, that was sucessively passed in McCoy cell line was used. Infected cells from several passages maintained in Eagle's medium supplemented with 2% of foetal calf serum (FCS) showing titres that ranged from $10^{4.5}$ to 10^{8} LD50%/ 0.03ml, were inoculated into the brains of newborn mice and the death rate was calculated by the Reed-Muench[10] method. These cells were frozen and thawed for three times and the cellular fluid was collected and processed according the diagram.

VIRUS PURIFICATION: The infected cellular fluid, after the freezing-and-thawing process, was centrifuged at 1000 x g for 20 minutes. The supernatant was poured and added of zinc acetate until reaching a 0.02 M. The pH was adjusted to 6.8 and the supernatant was kept at 4°C of temperature and it was centrifuged again at 1000 x g 40 minutes. After that, the supernatant was discarded and to the precipitate was added a tris-phosphate EDTA 0,02 M Solution with pH = 7.8 (NTE) (1/80 of the original volume of the infected fluid). After centrifugation, the pellet was discarded and the supernatant was filtered though a Sephadex G-75 column.

GEL FILTRATION: All samples were tested for molecular integrity by two collumns with 1.5 cm diameter and 50 cm of height were prepared for gel filtration. The first filtration was through Sephadex G-75 and the second was through Sephadex G-50: both were treated with tris-phosphate 0.02M at pH=7.8 (NT). Fractions were colleted by continuos gravity current and the Sephadex G-75 filtration evidenced only peak between the fractions of the tubes 5 and 10. This peak was concentrated with the help of Amicon system of membranes and then went through the Sephadex G-50 collumn.

SACHAROSE GRADIENT: The peak obtained by Sephadex G-50 collumn was concentrated through a saccharose gradient (10% to 60%) also buffered with tris- phosphate and centrifuged in a Beckman spin rotor 25.1. to 61.000 x g during 40 minutes at 4°C.

CROSSED IMMUNOELECTROPHORESIS: This technique was made during all the purification process with the objective of following each on the purification phases.

In the crossed immunoelectrophoresis the first step was carried out using agarose gel (medium type from Sigma Labs.), melted in a tris-barbiturate 0.02M, pH = 8.6 buffer.

The same buffer was used in the electrophoresis cube. The voltage was constant during the process and was 8-10 V/cm. During the first run, the duration was 1:30 hours and during the second run, performed across the first, with the hyperimmune serum containing in the gel, the duration was 3:00 hours.

Then the slides were washed in saline 0.15M several times in order to eliminate non preciptate serum and fixed in an ethanol/ methanol (50/50) solution. The slides were stained with an ethanol acetate acid/ brilliant Comassie blue G (500/500/0.5g) . To avoid excess of staining the slides were washed with solution containing ethanol/ acetic acid/deionated water (45/10/45) and were allowed to dry. The preciptated fractions could be seen, then , following the technique originally described by Week[14] .

GEL ELECTROPHORESIS (SDS/PAGE): The obtained peaks in two gel filtrations were submitted to an electrophoretic run in order to observe the polipeptides of the samples. Each sample was mixed (v/v) to the lysis buffer (1% Sodium Dodecyl Sulfate + 1% beta mercaptoethanol + 0.1% bromophenol blue). Each mixture was boiled to 100°C during 1 minute. The lysis product of each mixture was transfered in poliacrylamide gel prepared in a gradient varying from 7.5% to 12%, according Laemili's[4] technique.

IMMUNOENZIMATIC TEST: It was performed to verify the recovery of the viral activity after concentration in the saccharose gradient.

The opalescent ring that is shown in a belt aproximately 45% from the top of gradient was collected and dialysed against decreasing concentrations of saccharose (mol/liter). The final volume of 30 ml after dialysis, was divided into thirty/ 1 ml sample and they were tested against anti-rabic serum to detect viral activity.

It was used a flexible plastic plate with 96 holes, in each of them it was poured 15 ul of each one of the thirty fractions and the plate was maintened overnight ar 4°C.

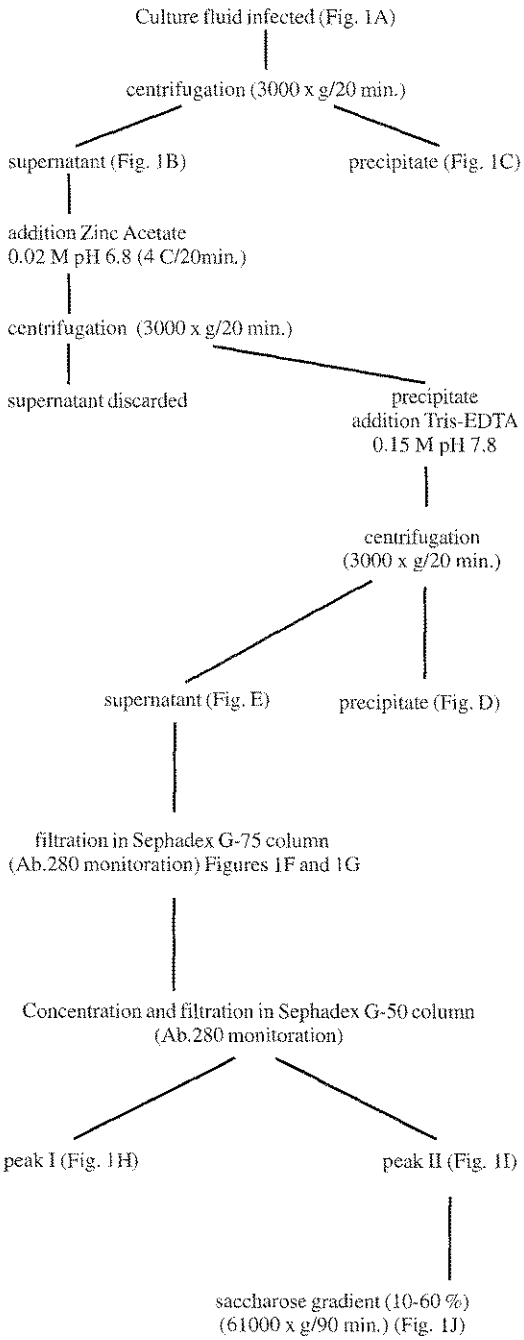
For the reaction antigen-antidoby 15 ul of anti-rabic serum was added and the plate was incubated at 37°C during 45 minutes, then the supernatant was discarded with the help of an adequate aspirator and the material was washed three times with buffered saline plus 0.03% of Tween (tensioactive agent); the excess of liquid was discarded and anti-IgG conjugate bound to peroxidase was added. The plate was incubated at 37°C for 30 minutes in a umid chamber and then washed again three times. Next step was additioned the cromogenic substrate, orto-phenildiamine (OPD) diluted in citrate buffer pH 5.4 and oxigenated water 0.03 v/v. As the enzimatic reaction was completed it was stopped with a sulfuric solution 4 n and the interpretation was done in an ELISA reader (wavelenght 492nm). This test was made in triplicate and holes were used for controls.

RESULTS

Each step of the purification process as presented in the Diagram 1 was followed by crossed immuno-electrophoresis technique.

The Figure 1A shows the beginning of the process: collected cellular fluid from several passages of rabies virus (ERA strain) in the McCoy cells.

DIAGRAM OF VIRAL PURIFICATION



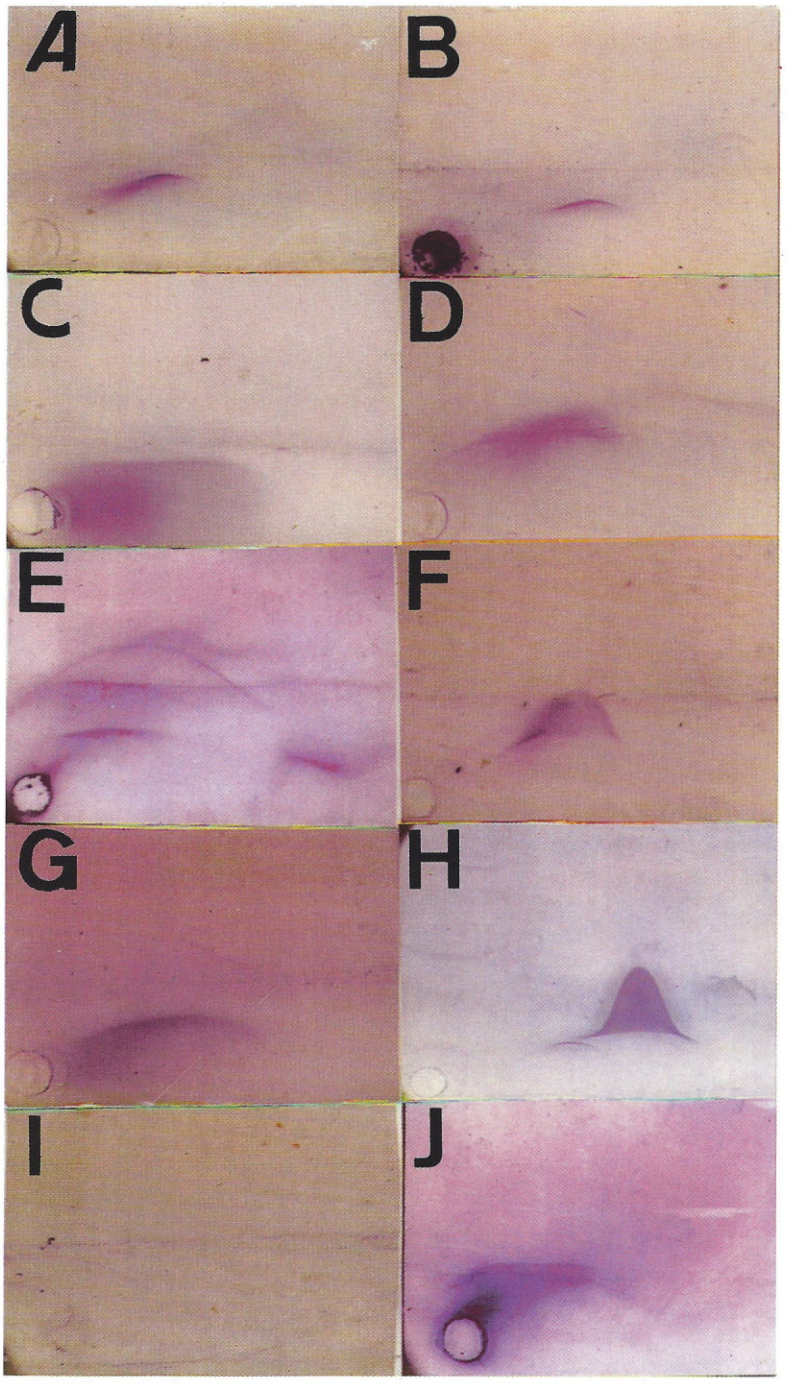


Figure 1: Crossed immunoelectrophoresis of each step of the purificatio process according to the Diagram.

1A: Infected cellular fluid;
1B: Supernatant after the first centrifugation;
1C: Preciptate from first centrifugation discarded;
1D: Preciptate from the third centrifugation (discarded);
1E: Supernatant before filtration in Sephadex G-75 column;
1F: Elution product of Sephadex G-75 column;
1G: Retained material in the column (eluate);
1H and 1I: Peaks I and II collected after filtration in Sephadex G-50 (280 nm of absorbance).
1J: Peak II after concentration in saccharose gradient.

According to Diagram 1 the first step of the process has eliminated all cellular debris. Figure 1B shows the supernatant after centrifugation at low speed: the preciptate (Figure 1C) was discarded.

Figure 1D is the preciptate obtained after the centrifugation obtained after preciptation with zinc acetate diluted in NTE buffer, while the Figure E represents the suppernatant, which was eluted through Sephadex G-75 column (Figure 1F). This results is also show in the Figure 2. Figure 1G is retained material in Sephadex G-75, that is the elements of high molecular weights.

The elution product was concentrated by ultrafiltration with membranes of 0.015 µm of porosity. The volume was reduced to 10% of the initial volume and then, the obtained product was eluted through Sephadex G-50 column. The eluted product show two well defined peaks at 280nm of absorbance (Figure 3, peak I and peak II), which are equivalent to Figure H and Figure I, respectively.

The Figure 4 shows the electrophoretic profile where lines 1 and 2 correspond to the patterns of molecular weights. Line 3 is product of peak I elu-

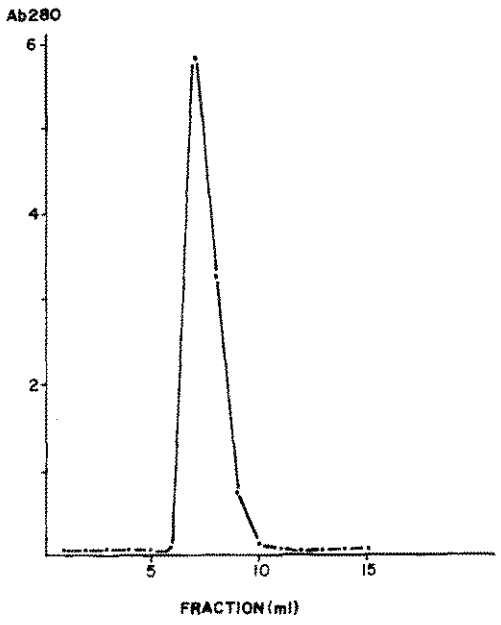


Figure 2: Cromatography in Sephadex G-75. System: Column (1.5 x 50 cm) eluted NTE buffer. Fraction collected (3 ml), collected (3 ml), monitored by Absorbance 280 nm.

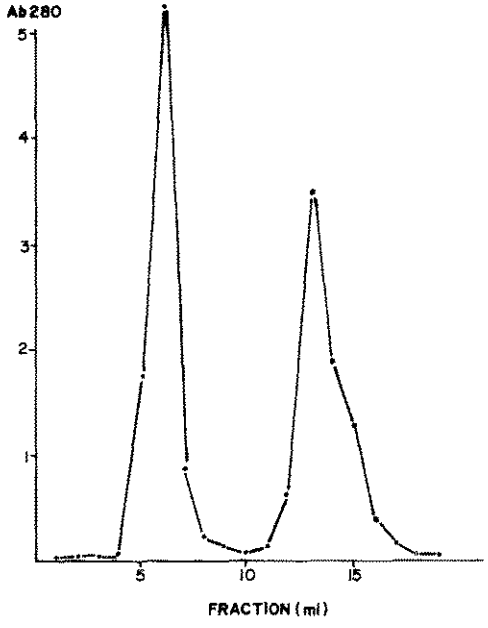


Figure 3: Cromatography in Sephadex G-50. System: Column (1.5 x 50 cm) eluted NTE buffer. Fraction collected (3 ml), collected (3 ml), monitored by Absorbance 280 nm.

tion and line 4 is the product of peak II while line 5 is peak II after concentration in saccharose gradient (equivalent to Figure J.)

In line 3 it can be observed one band with the same molecular weight of albumine that correspond to peak I and Figure 1H. In line 4 the presence of two bands

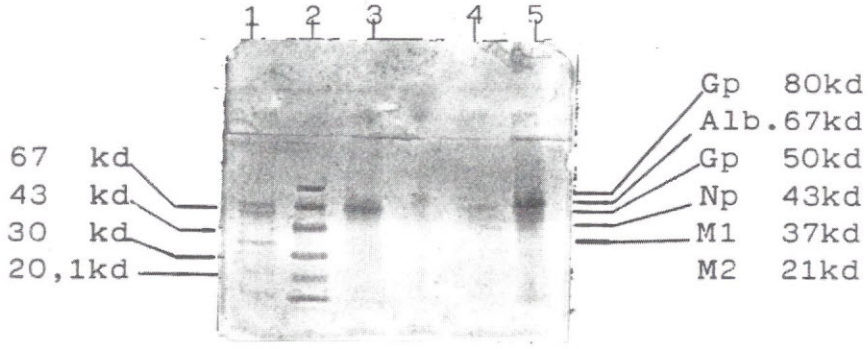


Figure 4: Electrophoretic separation in poliacrylamid gel SDS-PAGE.

Lane 1: high molecular weight
Lane 2: low molecular weight
Lane3: Peak I (Sephadex G-50)
Lane 4: Peak II (Sephadex G-50)
Lane 5: Peak II after concentrarion in saccharose gradient.

is observed, one more strongly stained with molecular weight near that of the albumine and other band weakly stained, that is difficult to see, with molecular weight around 50k daltons. However, in line 5, it can be better observed the concentrated material where several bands with similar molecular weight to that rabies of virus proteins appear: the glycoprotein (Gp) of 80 and 50k daltons, the nucleoprotein (N) of 43 k daltons and the proteins of the membrane (M1 and M2) with 37 and 21k daltons besides others proteins of low molecular weights but also much diluted.

DISCUSSION

In this work the purification of rabies virus multiplied in McCoy cell line was demonstrated and the caracterization of biochemical and immunological aspects of viral fraction purified through the crossed immunoelectrophoresis following Week[14] is showed.

Sokol's[11,12] classical method was used for the purification with some modifications: the treatment with DNase and RNase after elution in Sephadex G-75 columm was eliminated and it was introduced another elution with Sephadex G-50 instead of enzimatic treatment aiming at the elimination of the albumine fraction.

Neurath[6] et alii has demonstrated that rabies virus presents glycoproteins with 50 k daltons of molecular weight ; these glycoproteins are located in the spikes present in the viral envelope and are responsible for the immunogenicity of rabies virus.

Dietzschold[2] has isolated and has purified the glycoprotein located in the external wall of the virus membrane with help of a Sephadex G-200 column. He obtained a glycoprotein of 400k daltons of molecular weight "in natura". After a denaturation with detergent, Nonidet p40, and electrophoretic separation in poliacrylamid gel SDS-PAGE it became demostrated that this glycoprotein consisted of two chains of glycoproteins, one with molecular weight of 80k daltons and another with 50k daltons.

During this work, after the purification two proteins of molecular weights similar to that glycoprotein G(80k daltons and Gp 50) were evidenced and also contamination of bovine albumine fraction with molecular weight of 67k daltons from the serum and another components of the virion as nucleoprotein N (43k daltons) and membrane proteins M1(37 k daltons) and M2 (21k daltons).

After the electrophoretic separation on poliacrylamid gel SDS PAGE it is difficult to identify whith band correspond to Gp 50 or albumine, due to nearness of these two bands. However, with the crossed immunoelectrophoresis, technique this caracterization became possible. As it can be can observed in Figure 1H, according to Nogueira et alii[7] that characterized the imunogenic fractions of albumine and that of the viral fraction of the Fuenzalida - Palacios vaccine, the electrophoretic profile of albumine and that of the viral fraction are well defined.

The introduction of gel filtration in Sephadex G-50 showed efficiency in the resolution of both peaks, separating the albumine in peak I, thus making possible the elimination of the undesired contaminant, for the albumine is highly immunogenic during the immunization process. However, it is known that albumine protects the maintenance of viral titre during stockage at low temperature. So, it became possible to maintain the virus protected for long time, being this one of the adaptations to conditions of work in the laboratory.

Figure1J shows crossed immunoelectrophoresis after concentration in saccharose gradient: it can be ob-

served two lines of precipitation what is in accordance to Neurath's[6] findings: he also noticed that, after the saccharose gradient , two lines of preciptation were observed in the Outcherlony technique.

However, in Figure 1I only one line of preciptation can be seen and it corresponds to peak II before concentration in saccharose gradient. In Figure 1J the precipitation main line presented an electrophoretic mobility distinct from its equivalent in Figure 1I . This result suggests that the sacchorose gradient clumped particles that were dispersed and, due to the molarity variation in the gradient, the electrophoretic mobility probably could be altered .

In Whitt et alii[15] studies it became proved that the ionic strenght of the buffer in saccharose gradient turns the glycoprotein olygomer unstable causing the dissociation and solubilization of the glycoprotein. According to the same authors the olygomer should have a molecular weight upper 200k daltons: as was observed by Dietzschold[2] this olygomer is composed by three parts forming a dimer with molecular weight aroud 80k daltons and a monomer with molecular weight around 50k daltons, modiffing the oligomeric structure of the virus glycoprotein, but it is important to notice that the sequence of the quartenary structure of the extra-celular dominium of the glycoprotein , exactly what gives immunogenicity, is not alterated.

This methodology allowed the recovery of rabies virus from McCoy cells culture, purified and free of contaminants or other cellular proteins, even considering that this purified fraction of the virus has not been tested in relation to its capacity of inducing protection or immunity.

Atanasiu et alii[1] reported in relation to a comparative study was made with vaccines obtained from three different cell lines, was demonstrated that, after purification of each vaccine the greatest part of the contaminants was eliminated but not those of cellular proteins. Perrin[13] discussed that in the purification processes, besides cellular contaminants some active viral particles and some epitopes (soluble glycoproteins) are lost.

However, the viral recovery was verified during the immunoenzimatic test by the viral activity: data not showed .

This study has demonstrated the antigenicity in rabies virus recovered in McCoy cell line. A favourable aspect of this preparative method was that we have a quantitative notion of the purified product and the level of the contaminants be maintained at the minimum possible.

This methodology enables a production process of the viral bulk in large scale and makes possible all process monitoration by an efficient not expensive method, allowing adequate quality control of the product.

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RESUMO: O vírus rábico replicado e liberado em cultura de células McCoy crescidas em meio suplementado com soro fetal bovino foi purificado de acordo com o classico método de Sokol[11,12], porém, submetido a algumas modificações.

Introduzimos a filtração em Sephadex G-50, em seguida a filtração em Sephadex G-75, eliminando o tratamento enzimático com DNase e RNase.

A eluição em Sephadex G-50 apresentou dois picos com atividade em 280 nm. O pico I continha soro albumina bovina e no pico II a maior parte da fração viral purificada livre de contaminantes como proteínas séricas ou celulares.

O pico I foi submetido à concentração em gradiente de sacarose, agrupando todos os componentes da partícula viral, sendo estes componentes caracterizados, bioquimicamente, através de separação eletroforética SDS-PAGE e imunoeletroforese bidimensional.

A imunoeletroforese bidimensional foi utilizada para acompanhar todo o processo de purificação, permitindo observar e visualizar quais frações foram eliminadas em cada etapa do processo.

Neste caso dupla vantagem verificou-se ao eliminar-se a soro albumina bovina com a introdução da segunda filtração em Sephadex G-50 e, conseqüentemente, a visualização através da técnica empregada, a imunoeletroforese bidimensional.

Esta abordagem é interessante nos processos de produção de antígenos purificados em larga escala, pois permite controlar o grau de pureza e obter um bom controle de qualidade do produto.

DESCRITORES: Vírus rábico, células McCoy, purificação, eletroforese bidimensional e glicoproteína.

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