Analysis of Alternative Purification of Beta-Propiolactone Inactivated, Tangential Flow Filtration Concentrated Vero Cell Derived Rabies Vaccine

Jagannathan Selvaraj”, Mani Kavaratty Raju1,2 and Vijayaraj Kumar Rajendran4

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Abstract

Rabies virus is a single stranded negative sense RNA virus that belongs to the genus lyssavirus of the rhabdoviridae family which causes an acute disease of vertebrate animals. In the production of vaccines, purification plays a crucial role in the vaccine efficacy. Viral inactivation and removal steps are critical parts in mammalian cell culture derived biotechnology products. Regulatory agencies are concerned with the presence of endogenous or adventitious agent in the cell lines or raw materials employed to manufacture pharmaceuticals protein from cell culture. Due to this, numerous techniques are affianced in the downstream processing of viral vaccines manufacturing especially chromatography. Advances in vaccine manufacturing have created an increasing demand for large volumes of highly purified viral antigens. Cellufine sulfate Chromatography which has been used for other viral vaccines of both animal and human, was used in this study for optimal recovery of valuable rabies viral protein and optimal removal of impurities such as residual cellular DNA and host cellular BSA successfully as a replacement of the existing zonal centrifuge method.

Keywords: Rabies; Chromatography; Cellufine sulfate; Residual DNA

Abbreviations

SRID: Single Radial Immunodiffusion Assay, TFF: Tangential Flow Filtration; ODD: Ouchterlony Double Immunodiffusion; HCP: Host Cellular Protein; HAU: Hemagglutination Unit

Introduction

During the production of viral vaccines the chromatographic techniques are vital part in protein purification; final product of these technique should be free from contaminants with higher purity. Selection of appropriate chromatographic matrix is very much reliant to the physiochemical attributes of target product. For the large scale downstream purification the flow rate plays a main role, it is more significant to other parameters [1]. The gram negative bacterial spores outer membranes (endotoxin) represents a major potential contaminant, which may cause nonspecific, their exclusion from immunobiochemical products, through apt purification procedures represents a major assignment of vaccine manufacturing process [2]. The Cellufine matrix was used for various proteins purification like respiratory syncytial virus (RSV) and vesicular stomatitis virus (VSV) [3], Helicobacter pylori urease enzyme [4].

Materials and Methods

Cells and virus

Vero cells (CCL-81) were obtained from ATCC at the passage number 125 as monolayer frozen cells and propagated with appropriate medium [5]. Further it was revived, sub cultured and seeded into roller bottles (2.5 × 10^6 cells/mL) with serum containing medium (SCM) and serum free medium (SFM) [6]. Further the cultures were incubated at 37°C with rpm of 0.6 rpm. Fixed strain of Pasteur virus (PV-11) was obtained from Institute Pasteur France and it was propagated, infected with monolayer vero cell line and the virus infectivity was analyzed [5,7]. The replicas infected vero cell supernatant (viral harvest) was collected and replenished with maintenance medium which contains only essential and non-essential amino acids along with meager amount of newborn calf serum which is for only in...
the function of maintaining the cell viability without cell proliferation. The challenge virus stranded strain was propagated in mouse brain and then titrated, it was used for RFIT and intracerebral rabies virus challenge. At every interval of 72 hrs for 5 harvests, infectivity in mice (LD50) was analyzed by in vivo through inoculating increasing dilutions of the virus material to Swiss albino and based on mortality in different dilutions [8] and viral titer was calculated [9].

Concentration and inactivation of rabies virus

Those viral harvests which have passed the in process quality control tests subsequently process for tangential flow ultrafiltration (CON), BPL inactivation [5], the SCM and SFM originated rabies virus, BPL inactivated concentrate was tested for its status by in vivo and in vitro method [10].

Purification strategy of rabies virus

Matrex Celluline Sulfate (Millipore) was used as media for affinity chromatography purification, which was immobilized with 100 mM of NaCl in 10 mM PBS (buffer A), pH 7.6. For purification step gradient buffers used with increasing concentration of NaCl (300 (buffer X), 600 (buffer B), 1200 mM (buffer C) and 1800 mM (buffer D)) 10 mM PBS, pH 7.6 was used. The diameter and height of the column was 11 mm, 250 mm respectively, the I.D/O.D of the flow tubes are 1 mm/1.6 mm, the bed support was 10 micron polypropylene layer. The Celluline sulfate was loaded and packing flow rate was maintained until reaching the bed heights (6, 7, 8, 9, and 10 cm) used with matrix in different volume such as 12.5, 14.3, 15.6, 17.1 and 18.7 mL, all the experiments were conducted in ambient temperature. In SCM originated rabies viral, the protein concentration was 473 µg/mL, mouse infective titer was 10^4.5, no visible zone between rabies antigen and antibodies were found in SRID, the HCP and Residual cellular DNA were 500, 10 ng/mL, respectively, the CON was further diluted in different volume such as 12.5, 14.3, 15.6, 17.1 and 18.7 mL, all the experiments were conducted in ambient temperature. In SCM originated rabies viral, the protein concentration was 473 µg/mL, mouse infective titer was 10^4.5, no visible zone between rabies antigen and antibodies were found in SRID, the HCP and Residual cellular DNA were 500, 10 ng/mL, respectively, the CON was further diluted in buffer A prior to loading. In each experiment 15 fractions were collected, step recovery was calculated. The eluted fractions were pooled and desalted by diafiltration.

Formulation and Quality attributes of rabies virus

The desalted proteins are formulated with human albumin and maltose as and it was subjected to their quality attributes, sterility test, pH, abnormal toxicity, and innocuity was done [5]. The single human dose was used for immunogenicity analysis. The total protein was measured by using BCA Protein Assay Kit [11]. The HA test was done as per the technique described by Kuwert et al. with modifications; the end point result was taken by the highest virus dilution giving detectable HA [12]. The HA was expressed in HA units by calculating OD at 260 nm and 280 nm. The peak analysis of viral protein recovery and the OD at 260/280 nm. The peak analysis of viral protein recovery and the removal of impurities had the symmetrical peak, as reflected in the OD at 260/280 nm. The peak analysis of viral protein recovery and the removal of impurities had the symmetrical peak, as reflected in the OD at 260/280 nm. The peak analysis of viral protein recovery and the removal of impurities had the symmetrical peak, as reflected in the OD at 260/280 nm. The peak analysis of viral protein recovery and the removal of impurities had the symmetrical peak, as reflected in the OD at 260/280 nm.

Results and Discussions

Optimization of laboratory scale chromatography for rabies virus purification

The concentrated, BPL inactivated rabies viral harvests were further purified with Celluline sulfate packed column chromatography on experimental basis. When the matrix column of 8 cm (15.6 mL of bed volume of matrix) was used, in case of purification peak analysis impurities were found to the symmetry, indicating the maximal recovery of viral antigen and the removal of maximal quantity of impurities, for the purification of P or B from Nesseria meningitidis they are used linear gradient buffer and the purity is higher in status [18]. The complete profile of affinity chromatography using cellulose sulfate is presented in Figure 1. The majority of impurities like HCP, cell metabolites, and host cell- DNA that not bound with matrix during adsorption with buffer A. Total of 15 eluted fractions were collected, Fractions 1 to 6 corresponds to buffer A, 7 and 8 corresponds to buffer B, Fraction 9 and 10 corresponds to buffer B, fractions 11 and 12 corresponds buffer C, the remaining fractions 13, 14 and 15 correspond to buffer D, each fractions are analyzed for OD at 260/280 nm. The peak analysis of viral protein recovery and the removal of impurities had the symmetrical peak, as reflected in the A260/A280, further it was confirmed with SRID unitage Table 1. Two isolates of street rabies viruses were found to have elution points at 0.2 M and 0.3 M KCl respectively in the type 20 CM-cellulose and type 20 standard grade DEAE celluloses purification an increasing the salt concentrations to 0.5 M KCl reduced trailing and resulted in higher titer of the street viruses [19].

| Height of the Matrix | Volume of the matrix (mL) | NaCl strength (Elution) | Peak analysis | OD at 260 nm | OD at 280 nm | SRID Unitage |
|----------------------|---------------------------|------------------------|---------------|--------------|--------------|--------------|
| 6 Cm                 | 12.5                      | 300 mM Symmetry        | 0.0106        | 0.0249       | 4.4          |
|                      |                           | 600 mM Asymmetry       | 0.1204        | 0.1773       | 4.64         |
| 7 Cm                 | 14.3                      | 300 mM Symmetry        | 0.2008        | 0.3175       | 6.5          |
|                      |                           | 600 mM Asymmetry       | 0.2219        | 0.3228       | 7.1          |
| 8 Cm                 | 15.6                      | 300 mM Symmetry        | 0.1904        | 0.1771       | 8.32         |
|                      |                           | 600 mM Symmetry        | 0.1520        | 0.2238       | 7.1          |
| 9 Cm                 | 17.1                      | 300 mM Symmetry        | 0.2669        | 0.2647       | 7.1          |
|                      |                           | 600 mM Asymmetry       | 0.3881        | 0.3971       | 8.2          |
| 10 Cm                | 18.7                      | 300 mM Symmetry        | 0.2557        | 0.4243       | 8.32         |
|                      |                           | 600 mM Asymmetry       | 0.3109        | 0.4849       | 8.2          |

Table 1: Analysis of optimal bed height for rabies virus purification with Celluline Sulfate. *Results are the mean values of 3 separate experiments.
When the bed height was increased from 8 cm to 9 cm and 10 cm the elution peak from the viral antigen was symmetry but the elution peak for removal of the impurities was found to be asymmetry. The corresponding SRID unitage revealed that the concentration of impurities remained to be on the higher side (SRID unitage 8.23 IU/ml in each case) and required further removal in order to render the final rabies vaccine passing the quality control tests.

**Yield and recovery analysis of Rabies viral protein**

The quantum of rabies viral protein during primary run and secondary run on the pooled material from primary run fractions was given below the Table 2. During the elution with X and B the column was washed until it reaches the baseline. A total rabies viral protein of about 455 mg/ml was subjected to elution with 300 mM, 600 mM and 1200 mM of NaCl in 10 mm of PBS. During the primary run the quantum of rabies viral protein recovery was found to be 87%, 10% and 0.37% respectively. However for maximal viral proteins, the optimal level of elution buffer was found to be 300 mm.

| Chromatography Purification Status | Sample | HAU per 0.2 ml | Total protein µg/ml | SRID Unitage | Level of impurities of Host cellular BSA | Host cellular DNA |
|---|---|---|---|---|---|---|
| Before* | TCAR-CON (SCM) | 16.5 | 37 | No visible zone | >500ng/mL | 10 ng/mL |
| | TCAR-CON (SFM) | 20 | 39 | No visible zone | >500ng/mL | 10 ng/mL |
| After* | TCAR-CON (SCM) | 1442 | 736 | 7.94 | Below 10 ng/mL | 0.01ng/mL |
| | TCAR-CON (SFM) | 1532 | 755 | 8.0 | Below 10 ng/mL | 0.01ng/mL |

Table 3: Status of the rabies virus after purification. *Results are the mean values of 3 separate experiments. TCAR: Tissue Culture Anti Rabies, CON: Concentrated rabies viral harvest; SCM: Serum Contained Media; SFM: Serum Free Media.

**Table 2: Rabies viral protein yield analysis in laboratory scale affinity chromatography.** *Results are the mean values of 3 separate experiments.

| Primary run | Concentrated, inactivated rabies viral protein | Chromatographic fractions
|---|---|---|
| Total protein mg/ml | 455 | 398 | 47 | 1.7 |
| Yield % | 100 % | 87% | 10% | 0.37% |

The HAU/0.2 ml, total protein µg/ml, SRID unitage IU/ml, HCP ng/ml and residual cellular DNA ng/ml before and after purification are given in the table 3 and the values was found to be, 16.5/0.2 ml, 37 µg/ml, no visible zone, around 500 ng/ml and around 10 ng/ ml respectively, for the concentrated material prepared out of SCM. After purification, these values were found to be in the order of 1/442/0.2 ml, 736 µg/ml, 7.94 IU/ml, 32 IU/ml, less than 10 ng/ ml and around 0.01 ng/ ml respectively. In case of SFM the concentrated material had HAU 20/0.2 ml and total protein was 39µg/mL; however the other values found to be same as in case of SCM. After purification these values were found to be 1532 HAU/0.2 mL, 755 µg/mL of total protein, 7.65 IU/ml of SRID unitage, less than 10 ng/ml of HCP and around 0.01 ng/ml of residual cellular DNA (Figure 2).

**Figure 2: Slot blot hybridization for quantitative detection of affinity column purified test samples and Vero cell substrate DNA C1 to C6 DNA with 10 ng, 1 ng, 0.1 ng, 0.01 ng, and 0.001 ng and 0.0001 ng dilutions respectively.**

**Samples 1 to 12 various stages of vero cell rabies vaccine production by Cellufine Sulfate column chromatography**

1 and 2 SCM and SFM originated, concentrated and βPL inactivated rabies virus, 3 and 4 Primary run fractions of 100 mm NaCl, 5 and 6 Primary run fractions of 600 mM NaCl, 7 and 8 primary run fractions of 300 mm NaCl, 9 and 10 Secondary run fractions of 300 mM NaCl, 11 and 12 are diafiltred 300 mm secondary run fractions.
The present result supports cellufine sulfate could be an ideal matrix for rabies virus purification. The compatibility of phosphate buffer saline at pH 7.6 worked noticeably well for rabies virus purification and the gradient buffer with varying strength of sodium chloride provided evidence of compatibility for the sample and appropriateness in eluting bound rabies virus. After purification using with cellufine sulfate affinity chromatography the HCP and host cellular DNA were found to be low. In the studies of Reap et al. for purification of alpha virus particles through cellufine sulfate matrix, eluted with 250 mM, the purity of the material was within the WHO requirements [20].

However the rabies viral protein was retained to the maximum, both in case of SCM and SFM that was analyzed by SDS-PAGE and it showed a lot of protein bands mainly in the molecular weight of 120, 66 and 58 (KDa Figure 3) and also showed without changes of protein in SCM, SFM. Rabies virus consists of different proteins but five structural proteins are more significant [21]. Moreover the 300mM NaCl eluted primary, secondary fractions from SCM and SFM originated rabies viral proteins showed two bands, one of which correspond with 66KDa indicating the GI (glycoprotein 65KDa). Glycoprotein is the major immunogen to which neutralizing antibodies are produced after vaccination. The main striking feature of this 300 mm eluted fractions was that it also showed a uniformly stained background against which proteins appeared as faint thin lines. In case of concentrated rabies viral proteins of SCM and SFM originated the lane A& B showed smudged bands of proteins it reveals the partial purification after TFF.

### Table 4: Immunogenicity analysis of SCM and SFM originated rabies virus after purification through cellufine sulfate affinity chromatography.

| Vaccine details | No. of animals used | RFFIT | Rabies Neutralizing antibodies in IU/ml |
|-----------------|---------------------|-------|---------------------------------------|
| SCM originated, purified pooled material of 300mM fractions | 8 | <05 | <05 | 32 | 32-64 |
| SFM originated, purified pooled material of 300mM fractions | 8 | <05 | 16 | 32-64 | 64 |
| Local reference (HO23) | 8 | <05 | 16:32 | 32-64 | 64 |

The SCM, SFM originated cellufine sulfate purified pooled material of 300 mM fractions was subjected to RFFIT technique for the analysis of rabies neutralizing antibodies IU/ml along with local reference (HO23). The seroconversion rate of guinea pigs blood samples are above 0.5 IU/ml on the 7th day itself.

The immunogenicity of the SCM and SFM originated rabies vaccines was found to be comparable with HO23 that revealed in the ODD (Figure 4) [22]. The experiments of cellufine sulfate purified of vero-cell derived Japanese encephalitis vaccine by Sugawara et al., showed that almost all low-molecular weight impurities were removed by Sucrose-density gradient ultra-filtration and that the residual impurities are removed column chromatography [23].

M: Marker protein; A: Concentrated rabies viral protein SCM originated; B: Concentrated rabies viral protein SFM originated; C: Primary chromatographic elution (600 mMNaCl); D: Primary chromatographic elution (300 mMNaCl) (SCM); E: Primary chromatographic elution (300 mMNaCl) (SFM); F: Secondary chromatographic elution (300 mMNaCl) (SCM); G: Secondary chromatographic elution (300 mMNaCl) (SFM).

Figure 4: Ouchterlony double Immunodiffusion assay. A: Negative control; B: Guinea pig serum of SCM Originated Purified pooled material of 300mM fractions; C: Guinea pig serum of SFM Originated Purified pooled material of 300mM fractions; D: Guinea pig serum of Local reference (HO23) rabies vaccine; S: Chromatographically purified fractions (second run, 300 mM) of Rabies antigen.

It is an excellent alternative to sucrose gradient centrifuge and other complicated operations. It is ideal for depyrogenating virus and other microbial extracts because it does not bind endotoxins. It has been demonstrated by many workers that it can bind, concentrate and...
separate viral antigens from unwanted proteins [23]. 233 fold of viral cell proteins were eliminated during Cellulose sulfate chromatography and separahse 66F and high recovering of hemagglutinin activity and also the removal of total protein, HCP and DNA from Human influenza virus, the final purified, formulated product which fulfill with regulatory requirements for single strain influenza vaccines [24].

Conclusion

The objective of the study was to develop the alternative purification techniques for the zonal centrifugation those was used during the production processing of cell culture rabies vaccine. The content of the host cell derived DNA and contaminating proteins such as bovine serum albumin and vero cell proteins were found to be low which is quite below IP/WHO [25]. All the formulated rabies vaccines are passed pH, sterility, avirulence and virus amplification tests for the testing of reactogenic and protection. The HCP per dose was found to be 8.5ng, 10.5ng, 5.5ng and 6.5ng/dose for the lots A, B, C and D respectively against the admissible level of 50ng/dose. All the four lots contained less than 0.01ng/dose of residual host-cell DNA against the permissible level less than 10ng/dose.

The substrate impurities like host cellular proteins, residual cellular DNA was completely removed and it was ensured as these may lead to various types of adverse reactions. As per the regulatory norms [5,26], the final vaccine product should have β-propiolactone more than 1.3500 dilution and thiomersal not more than 0.01% w/v to prevent adverse reactions. And also absence of non-structural and non-immunogenic viral proteins is necessary to improve purity and efficacy of the vaccines [27].

All production parameters including the use of serum free media were optimized and standardized to produce large quantities high titer viral harvests. A new technique “Cellulose sulfate Chromatography” which has been used for other viral vaccines of both animal and man, was used in this study for optimal recovery of valuable viral protein and optimal removal of impurities, it is a simple, scalable and inexpensive technique, the results showed that the vaccines are highly immunogenic as well as the protective in mice it leads to the sufficient degree of protection after vaccination.

Ethical Approval

For conduction of all animal experiments, prior permission had been obtained from the Institution Animal Ethics committee and the animals were housed, maintained and experimented according to the institutional regulations in force.

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