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Rapid and reliable quantification of reovirus type 3 by high performance liquid chromatography during manufacturing of Reolysin®

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ABSTRACT

Reolysin®, a human reovirus type 3, is being evaluated in the clinic as an oncolytic therapy for various types of cancer. To facilitate the optimization and scale-up of the current process, a high performance liquid chromatography (HPLC) method has been developed that is rapid, specific and reliable for the quantification of reovirus type 3 particles. Using an anion-exchange column, the intact virus eluted from the contaminants in 9.78 min at 350 mM NaCl in 50 mM HEPES, pH 7.10 in a total analysis time of 25 min. The virus demonstrated a homogenous peak with no co-elution of other compounds as analyzed by photodiode array analysis. The HPLC method facilitated the optimization of the purification process which resulted in the improvement of both total and infectious particle recovery and contributed to the successful scale-up of the process at the 20 L, 40 L and 100 L production scale. The method is suitable for the analysis of crude virus supernatants, crude lysates, semi-purified and purified preparations and therefore is an ideal monitoring tool during process development and scale-up.

1. Introduction

Reoviruses are naturally occurring oncolytic viruses commonly found in the respiratory and gastrointestinal tracts of humans but known to have no association with any human disease. These viruses are non-enveloped, icosahedral in shape with 10 segments of double stranded (ds) ribonucleic acid (RNA) genome enclosed by eight different proteins in two concentric capsids. Human reovirus serotype 3 Dearing strain has been shown to kill cancer cells with activated Ras signaling pathways without infecting healthy cells and tissues [1]. It is currently in Phase I/II clinical trials for the treatment of various types of cancer under the tradename Reolysin® (Oncolytics Biotech Inc., Calgary, Alberta, Canada) and preliminary results were found to be promising [2,3]. To support the ongoing and future clinical programs, large amounts of Reolysin® are needed requiring that the current process be scaled up. The bottleneck in the process was associated with the downstream process (DSP), with an infectious particle final recovery of less than 5%. A major limitation to the efficient scale up of the DSP steps was the lack of a rapid, specific and reliable in-process analytical method to quantify reovirus total particles, involving infectious as well as non-infectious particles. The most commonly used method of quantification is by optical density measurements at 260 nm ($OD_{260\,nm}$) [4]. This method, however, requires highly purified reovirus material devoid of contaminants and therefore the quantification of crude lysates and semi-purified virus preparations is impossible. Presence of highly absorbing compounds at $OD_{260\,nm}$ (such as DNA) will result in an overestimation of particle concentration. Traditional assays for infectious titer quantification such as the tissue culture infective dose at 50% (TCID50) and the plaque-forming unit (pfu) require several days to obtain a result. In order to facilitate the optimization of conditions and future scale-up of the Reolysin® purification procedure, a high performance liquid chromatography anion exchange method was developed for reovirus total particle quantification using the UNO Q polishing column (Bio-Rad, Hercules, CA).

This paper presents the results obtained during the development and optimization of the method including characterization of the Reolysin® in-house standard and determination of optimum pH, NaCl linear gradient for virus elution, and sample volume for injection. Homogeneity determination by photodiode array (PDA) analysis of the eluted virus peak in crude and purified virus samples and the generation of the standard curve for quantification is discussed. Most importantly, the applicability of the method to

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quantify reovirus particles in culture supernatant, and in-process samples during the optimization and scale-up of the downstream process to manufacture Reolysin® is also presented.

2. Materials and methods

2.1. Reovirus production

Human embryonic kidney (HEK) 293 suspension (S) cells were cultured in a proprietary formulation cell culture medium (SAFC Biosciences, St Louis, MO). Reovirus production was performed by first seeding the HEK 293 S cells to a density of 0.4×10^6 cells/ml in half of the final production volume (i.e. 10 L for a 20 L production) then growing the culture to a density between 1.8 and 2.4×10^6 cells/ml at 37 °C, pH 7.2 with 40% dissolved oxygen and an agitation speed of 75 rpm. Fresh medium was then added to the production vessel for a final cell density between 0.9 and 1.2×10^6 cells/ml followed by virus infection at an MOI of 0.5. The infected culture was maintained under the same operating conditions and samples were collected for analyses at different hours post-infection (hpi). When the cell viability reached between 20% and 50%, Triton X-100 (SAFC Biosciences, St. Louis, MO) at a final concentration of 1% was used to lyse the cells for 1 h at 37 °C. Benzonase® (SAFC Biosciences, St. Louis, MO) at a final concentration of 10 U/ml lysate was added thereafter. The post-lysis, post-Benzonase® virus lysate was cooled down to 15 °C *in situ* prior to further downstream processing.

2.2. In-house standard preparation

An in-house standard was generated according to the following purification process. In brief, 5 L of a virus lysate was clarified through an 8.0 μm polypropylene membrane, followed by a 3.0/0.8 μm cellulose acetate double membrane capsule (Sartorius, Edgewood, NY). The clarified virus lysate was concentrated and diafiltered against a 0.1 M phosphate buffer, pH 7.2 +5% glycerol using a hollow fiber cartridge with a 300 kDa molecular weight cut-off (GE Healthcare, Piscataway, NJ). The concentrated and diafiltered virus retentate was purified by ion-exchange chromatography (IEX) using a Q-Sepharose HP resin (GE Healthcare, Piscataway, NJ). The IEX eluted virus was further polished by gel permeation chromatography (GPC) using a Sepharose 4 FF resin (GE Healthcare, Piscataway, NJ) and the virus peak found in the void volume was filtered through a 0.22-μm cellulose acetate membrane (Sartorius, Edgewood, NY). Sucrose and $MgCl_2$ was added to the bulk of the final purified reovirus at a final concentration of 5% and 2 mM, respectively then aliquoted in 250 μl volumes into polypropylene sterile cryovials (Ultident, Montreal, Quebec, Canada) prior to storage at −80 °C. Although we have not seen any problems with non-specific binding using other plastics or glass, all storage containers used in this process were made of polypropylene material unless otherwise specified.

2.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot analyses

A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a ready to use 4–15% Tris–HCl gradient gel under reducing conditions (Bio-Rad, Hercules, CA) was performed to resolve the protein components of the reovirus. Silver staining of the gels (Silver Stain PlusKit, Bio-Rad, Hercules, CA) allowed visual detection of protein bands. To confirm the virus identity, western blot analysis was carried out using a Hybond ECL nitrocellulose membrane (GE Healthcare, Pistacaway, NJ) and a Trans-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA). A primary polyclonal antibody against reovirus (McKesson Bioservice, Rockville, MD) was used in combination with a rabbit anti-goat IgG peroxidase conjugate (Jackson Immunochemicals, Mississauga, ON). Detection was performed with a BM Chemiluminescence Blotting Substrate (POD) kit (Roche Diagnostics Corp., Indianapolis, IN) and the Kodak Image Station 440 CF (Mandel Scientific Co. Inc., Guelph, ON).

2.4. Negative stain electron microscopy

Negative stain electron microscopy (NSEM) was performed at the Institut Armand Frappier (IAF, Laval, QC, Canada) according to a method previously described [6]. Briefly, virus samples were diluted in 10 mM Tris, 1 mM EDTA, 100 mM NaCl (TEN), pH 7.5. Aliquots of the diluted sample were placed in 240 μl microtubes with Formvar and carbon coated grids inserted in the bottom of the microtubes. The tubes were placed in an Airfuge A-100 fixed angle rotor (30 °C) and centrifuged at 20,000 × *g* for 5 min. The grids were recovered and dried with bibulous paper then stained with phosphotungstic acid (3%, pH 6.0). The samples were visualized using the transmission electron microscope.

2.5. Total particle quantification by optical density measurement at 260 nm

The total particle concentration (VP/ml) of the in-house standard was quantified by adding SDS to a final concentration of 0.5% to the virus sample, then heated at 55 °C for 5 min and centrifuged at 11,750 × *g* for 1 min. The supernatant was transferred to a quartz cuvette and the absorbance at 260 nm was read. A phosphate buffered saline (PBS) buffer, pH 7.5 with no virus was used as a blank prior to reading the absorbance of the samples. The VP/ml was calculated by multiplying the absorbance by 2.1×10^{12} VP/ml [4].

2.6. Infectious particles quantification by 50% tissue culture infectious dose assay

The infectious particle concentration (TCID50/ml) of reovirus in crude, semi-purified and purified preparations was determined by the TCID50 assay [7] using L-929 cells (American Type Culture Association, Manassas, VA). In a 96-well plate, the cells were seeded at a density of 1×10^4 cells per well in 100 μl of JF10 media (Sigma–Aldrich Canada, Oakville, ON) and incubated overnight at 37 °C with 5% CO_2. The next day, the cells were infected with 100 μl of several dilutions of the virus from 1×10^{-4} to 2.05×10^{-12} and incubated in a humidified environment for 14 days at 37 °C and 5% CO_2. The cytopathic effect (CPE) of the virus was assessed based on the change in cell morphology relative to the control (media without virus). All virus samples were filtered through 0.2 μm, 13 mm Supor membrane Acrodisc syringe filter (Pall Life Sciences, Ann Arbor, MI) prior to the analysis. The assay was always performed in triplicates unless otherwise specified.

2.7. HPLC analysis

An HPLC Alliance System (Waters, Milford, MA) equipped with a 2690 separation module, an in-line degasser, 996 photodiode array (PDA) detector and Millennium^{32} software was used for data acquisition and peak integration. A UNO™ Q polishing anion exchange column, 4.6 mm × 10 mm (Bio-Rad Lab. Hercules, CA) was used for the virus separation. The mobile phase stock solutions used were as follows: (A) 0.25 M HEPES, pH 7.10 ± 0.1; (B) 2 M NaCl and (C) Milli-Q® purified water. All mobile phases were filtered through a 0.45 μm membrane before use. Prior to sample analysis, the column was equilibrated with a start buffer (20% A, 7.5% B and 72.5%

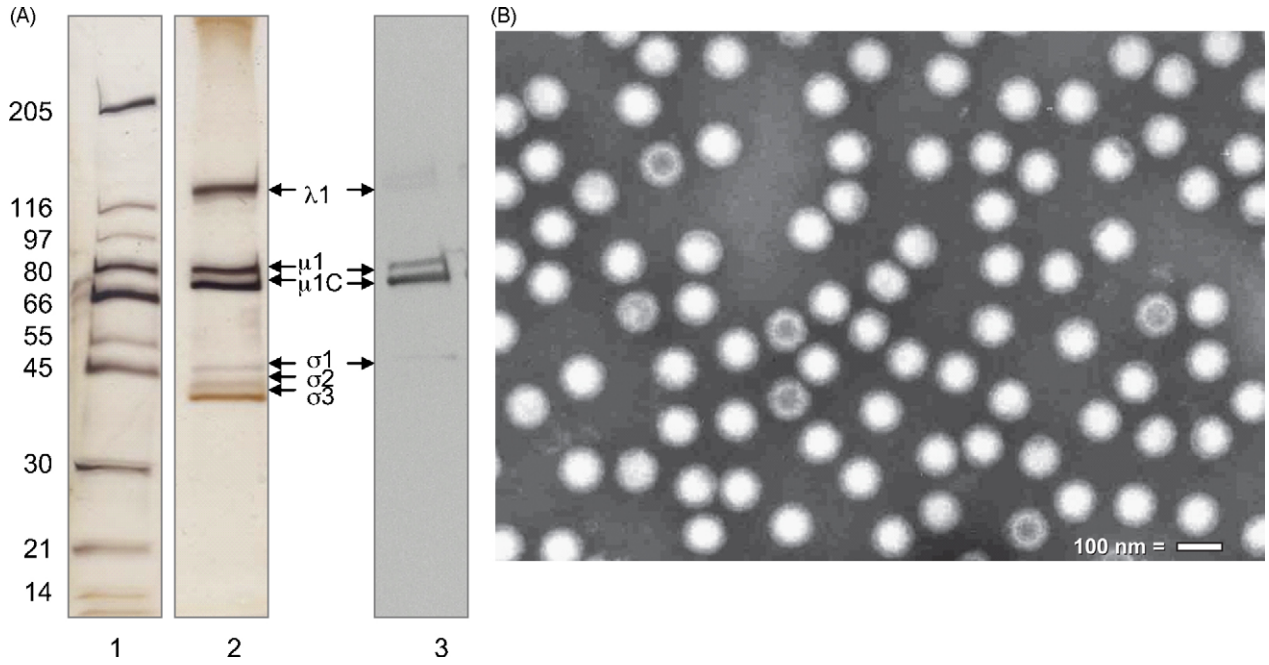

Fig. 1. (A) is the sodium dodecyl sulfate polyacrylamide electrophoresis of a Reolysin® in-house standard using 4–15% Tris–HCl ready gels with silver staining of the protein bands (lane 2) and western blot analysis (lane 3). The major viral proteins detected were λ1, μ1, μ1C and σ1–3 (lane 2) based on their apparent molecular weights using the broad protein molecular weight markers in lane 1. The μ1 and μ1C were clearly detected by western blot analysis using a polyclonal Ab against reovirus with less intense band of λ1 and σ3 (lane 3). Figure (B) is the negative stain electron microscopy and phosphotungstic staining of the Reolysin® standard. The preparation had about 78% of the particles in excellent (intact) form that ranged in size between 87 nm and 90 nm with an average of 88 nm.

C) for 10 min at 1 ml/min. Thereafter, three buffer blanks (50 mM HEPES, pH 7.10) were injected to ensure a flat baseline. All virus samples were filtered through 0.2 μm Supor membrane 13 mm Acrodisc syringe filters (Pall Life Sciences, Ann Arbor, MI) prior to the analysis. When required, samples were diluted after 0.2 μm filtration using the start buffer. After sample injection, the column was washed with the start buffer followed by virus elution with a linear gradient from 7.5% to 17.5% B in 20% A at flow rate of 1 ml/min. After the virus elution, the column was regenerated with 2 M NaCl for 4 min at a flow rate of 0.5 ml/min then re-equilibrated with start buffer prior to the next sample injection. Analysis was performed at ambient temperature (18–20 °C) with the samples kept at 4 °C in the sample chamber.

2.8. Photodiode array peak purity analysis

The purity of the reovirus peak was analyzed using the PDA and the Millennium[32] software. Using a broad spectrum of ultraviolet (UV) and visible (vis) wavelengths between 190 nm and 800 nm, the spectral differences at every data point across the entire peak was determined using the peak apex as the reference point. A peak is homogenous when the purity angle (°), defined as the weighted average of all the spectral contrast angles, is lower than the purity threshold angle, which in this analysis was based on the sum of assay noise and solvent contribution.

3. Results and discussion

3.1. In-house standard characterization

The in-house standard was demonstrated to be highly pure with the detection of only the major viral capsid proteins shown by SDS-PAGE analysis and silver staining of the gel (Fig. 1A, lane 2). The protein bands were identified based on their apparent molecular weights (MW) as compared to the broad MW protein markers in lane 1, and included: λ1 (120 kDa), μ1 (79 kDa), μ1C, (72 kDa) and σ1, σ2, σ3 (49, 46, 42 kDa, respectively). Reovirus type 3 consists of eight structural proteins namely λ1, λ2, λ3, μ1, μ2, σ1, σ2, and σ3. The proteins λ1, λ2, λ3, μ2 and σ2 are located in the inner capsid while μ1, σ1 and σ3 are located in the outer capsid. The separation of these proteins was described to be dependent on the type of SDS-PAGE gels and analysis conditions used [8]. Under the conditions used in this analysis, the λ1 protein could not be resolved from the λ2 and λ3 protein bands which is consistent with the results obtained by others using CsCl purified reovirus [9]. The virus identity was further confirmed by western blot analysis using a polyclonal antibody against reovirus with the major detection of the μ1 and μ1C proteins and minor detection of the λ1 and σ1 proteins (Fig. 1A, lane 3).

The morphology and quality of the in-house standard was determined by NSEM and showed that the size of the virus ranged between 87 nm and 90 nm with an average of 88 nm (Fig. 1B). About 78% of the virus particles showed intact forms. The rest of the particles were either completely broken or intact but immature virions lacking the viral genome.

This standard was highly infectious with a titer of 1.76×10^{11} TCID50/ml (mean of triplicate analyses) and 13% relative standard deviation (R.S.D.). The total particle concentration (mixture of both non-infectious and infectious particles) was 2.21×10^{12} VP/ml (mean of duplicate analyses) and 1% R.S.D. determined by the $OD_{260\,nm}$ method. Using the two methods described, the IVP/VP ratio of the preparation was 8%.

3.2. Reovirus peak identification

Reovirus type 3 particles have an isoelectric point (p*I*) of 3.9 [10]. At a pH above the p*I*, the virus has a negative charge and binds to an anion exchange column. To determine the salt concentration necessary to elute of the virus using the UNO™ Q polishing column, the in-house standard was initially separated upon sample

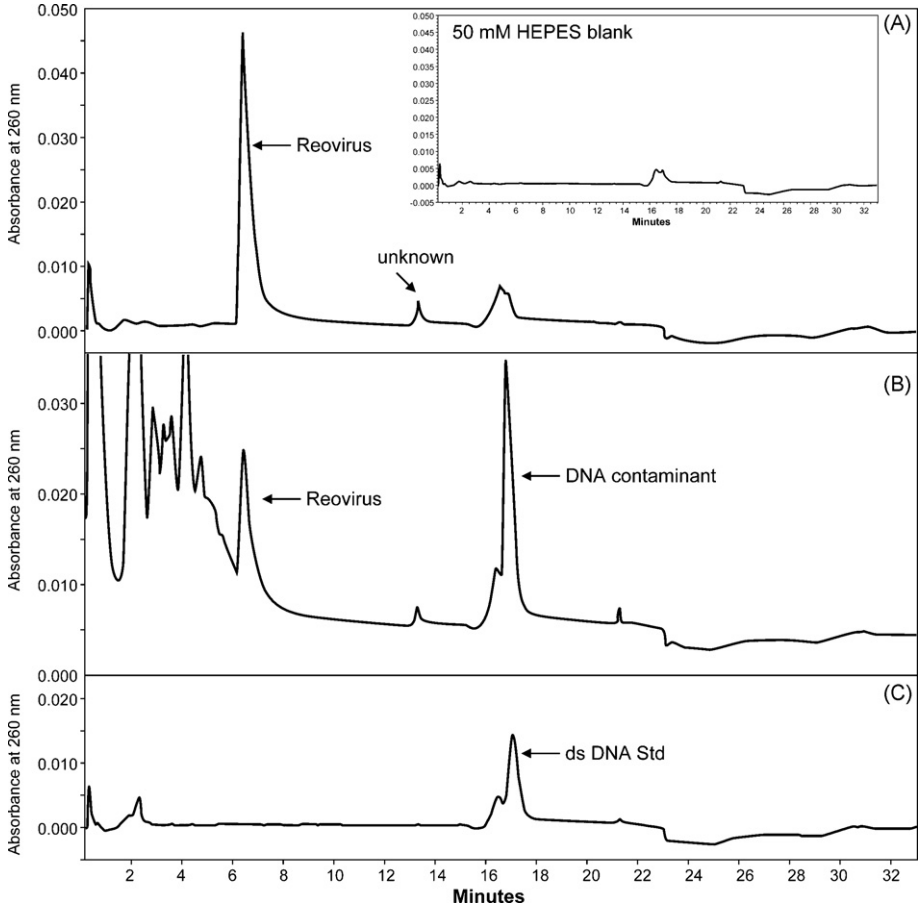

Fig. 2. Initial HPLC profiles of (A) an in-house standard, (B) virus lysate and (C) double stranded lambda (λ) DNA standard using a linear gradient elution from 0 mM to 500 mM NaCl in 50 mM HEPES buffer, pH 7.10. The inset (A) is the HEPES buffer blank (50 mM HEPES buffer, pH 7.10). The separation was performed using a UNO^{TM} Q polishing column, flow rate of 1 ml/min and sample injection volume of 50 μl.

injection of 50 μl with a linear NaCl gradient from 0% to 50% B in 20% A, 80% C, pH 7.10 for 9 min followed by a 4 min hold at 50% B. Thereafter, another linear gradient was performed from 50% to 100% B for 10 min followed by 5 min of column regeneration at 100% B and the column was re-equilibrated with 20% A, 0% B, 80% C for 7 min. A flow rate of 1 ml/min was used during the entire analysis time of 35 min. Fig. 2A shows the profile of the in-house standard. The reovirus (indicated by an arrow) eluted as a single major peak at 6.3 min and 17.5% B (350 mM NaCl). The minor peaks detected (except for the peak labeled as unknown) were shown to be interferences coming from the buffer reagent that was confirmed by analysis of a HEPES buffer blank (50 mM HEPES, pH 7.10) (inset figure). Fig. 2B is a virus lysate profile showing the inefficient separation of the reovirus peak (indicated by an arrow) from contaminants eluted at the beginning of the analysis and assumed to be host-derived proteins. The peak eluted after the reovirus at 16.4 min was identified to be a DNA contaminant and was confirmed by analysis of a double stranded (ds) lambda (λ) DNA standard (GE Healthcare, Pistacaway, NJ) (Fig. 2C). Because the virus in the lysate sample was not efficiently resolved from the contaminants, it was necessary to optimize the method and also to reduce the total analysis time.

3.3. Method optimization

3.3.1. Efficient virus separation

During the optimization of the method, a virus lysate sample and 20% of solution A were always used unless otherwise specified. To efficiently resolve the virus from the contaminants eluted at the beginning of the analysis (shown in Fig. 2B), the method started with a column wash after sample injection at 7.5% B for 5 min to pass the contaminants in the column flow through since a majority of contaminants elute at 150 mM NaCl. Thereafter, the virus was eluted using a linear gradient from 7.5% to 17.5% B for 5 min and a hold for 3 min using the same gradient then another linear gradient was performed from 17.5% to 100% B for 3 min to elute the DNA contaminants. The column was regenerated for 4 min at 100% B followed by column re-equilibration in 20% A, 7.5% B, 72.5% C for 5 min prior to the next sample injection. A flow rate of 1 ml/min was used during the 25 min of total analysis time except during the column regeneration when the flow rate was reduced to 0.5 ml/min. Fig. 3A shows the profile of a well-resolved reovirus peak from the protein and the DNA contaminants. The virus specifically eluted at 9.7 min in 350 mM NaCl. The majority of the protein contaminants eluted at the beginning of the analysis in 150 mM NaCl and the DNA contaminant eluted at 14.3 min in 1.2 M NaCl. Fig. 3B shows the profile of the in-house standard with the optimized method.

3.3.2. Optimum pH and NaCl gradient elution

The optimum pH for the virus elution was investigated by varying the pH of solution A from pH 7.1 to 7.0, and 7.2. The obtained mean retention time (RT) and peak area (PA) of the eluted virus using the different pHs described (including pH 7.1) was 9.7 min and 468712 with R.S.D.s of 0.5% and 6%, respectively. The results did not vary from each other.

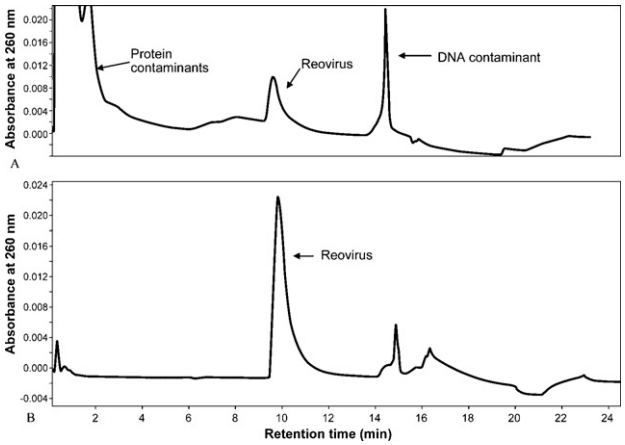

Fig. 3. HPLC profiles using the optimized method of (A) virus lysate and (B) Reolysin® standard with the optimized method. The separation was performed on a UNO™ Q polishing column, 0.16 ml, 50 μl sample injection volume, linear gradient virus elution from 150 mM to 350 mM NaCl in 50 mM HEPES, 7.10, flow rate at 1 ml/min for a total analysis time of 25 min. The reovirus peak was efficiently resolved from protein and DNA contaminants.

Using pH 7.0, 7.1 and 7.2, the optimum NaCl gradient elution for the virus was investigated using the elution gradients from 7.5% (150 mM) to 17% (340 mM) and 360 mM (18% B), respectively. A well-resolved virus peak was obtained with both gradients (profiles not shown). The virus RT and PA (including the 350 mM NaCl) did not vary, with R.S.D.s of 0.7% and 6%, respectively. At pH 7.0, 7.1 and 7.2, the virus therefore can be eluted using NaCl linear gradients from 150 mM to 355 mM without a significant change in the results.

3.3.3. Sample injection volume

Different injection volumes of 25 μl, 50 μl, 75 μl, 100 μl and 150 μl were also examined. A well-resolved virus peak was obtained with all the described injection volumes. The 25-μl injection, however, gave a high intra-variability (>20%) when virus samples with concentrations of $\leq 5 \times 10^{10}$ VP/ml were analyzed. The larger injection volumes (i.e. 100 μl and 150 μl) particularly with crude lysate samples was not desirable because of the need to do a frequent column cleaning due to high back pressure on the column. Moreover, such frequent cleaning with either 2 M NaOH, 2 M KCl or MeOH (as per the manufacturer's recommendations) resulted in a significant reduction of the virus peak area as well as inefficient peak resolution. In addition, in the case of the larger injection volumes, a minimum of three buffer blanks must be processed prior to the next sample injection to avoid carryover. Therefore, to maximize column longevity, and to avoid repeated buffer blank injections after every sample, an injection volume of 50 μL was preferred.

3.4. Peak purity by photodiode array analysis

For quantification purposes, the reliability of a method is also dependent on the purity of the eluted virus peak. With crude samples such as the virus lysate, a large amount of contaminants is expected and co-elution with the virus is probable and could lead to an overestimation of the results. With single wavelength detection, the presence of a co-eluent can only be evident if it is present in excessive amounts. Using PDA analysis, the in-house standard (representing the purified form of the virus) was first analyzed and showed a purity profile with an angle that was almost 0° across the entire peak (Fig. 4A). The purity angle was lower than the purity threshold suggesting a spectrally homogenous peak. At the point of the maximum impurity (M) that could possibly be detected, the purity angle (0.16°) was lower than the threshold angle (0.35°) confirming the homogeneity of the virus peak. Fig. 4B shows the peak purity profile of the reovirus peak in the crude lysate preparation. A similar profile (as the in-house standard) with a constant purity angle at 0° across the entire peak was observed. At the point of maximum impurity (*M*) that could possibly be detected, the purity angle at 1.02° was lower than the threshold angle at 1.52°, demonstrating a spectrally homogenous peak. The maximum wavelength of the reovirus peak in both virus preparations was also determined and found to exhibit multiple wavelengths at 230 nm and 260 nm, respectively (profile not shown). The 230 nm absorbance could be attributed to the capsid proteins while the RNA genome contributes to the majority of the absorbance at 260 nm. To confirm the purity of the eluted virus peak, the detection was performed using the dual wavelengths at 260 nm and 280 nm during the analysis of the in-house standard and the virus lysate samples. The absorbance ratio at 260 and 280 nm ($A_{260/280}$) by the $OD_{260\,nm}$ method is a commonly used method for purity assessment of purified virus stocks and has been used with cesium chloride (CsCl) purified reovirus virions [11]. The $A_{260/280}$ ratio of the eluted reovirus peak was 1.42 and 1.40 with the in-house standard and the virus lysate samples, respectively. These values were found to not differ significantly from the reported ratio between 1.37 and 1.41 of CsCl purified reovirus virions [11] confirming the purity of the reovirus peak with or without the presence of large amounts of contaminants.

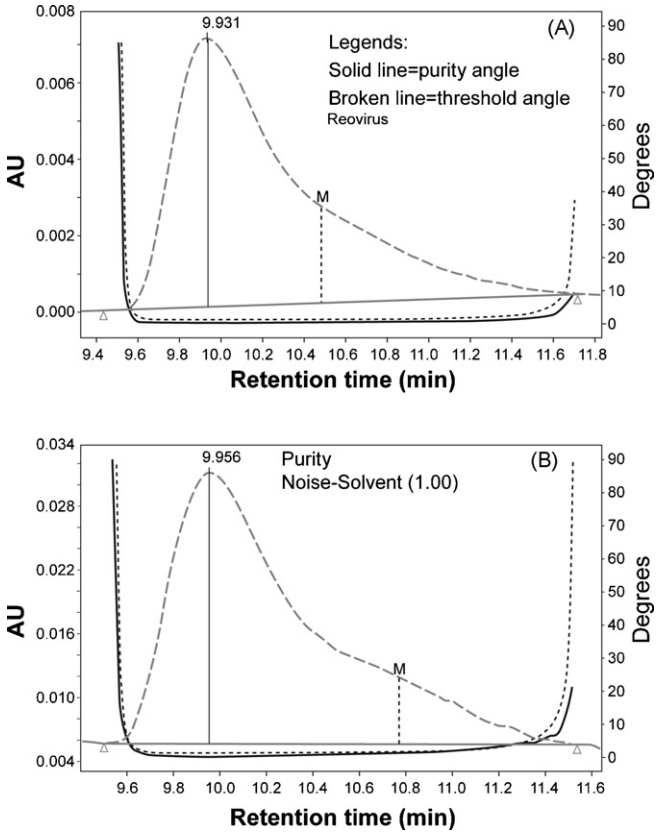

Fig. 4. Photodiode array analysis peak purity profiles of (A) in-house standard and (B) virus lysate. The virus peak was scanned using a broad spectrum of ultraviolet (UV) and visible (vis) wavelengths between 190 nm and 800 nm. The peak purity angle is represented by a solid line while the threshold angle by the broken line. The purity angle is lower than the purity threshold demonstrating a spectrally homogenous peak.

3.5. Standard curve

For the quantification of an unknown sample, a standard curve was generated using the in-house standard which was evaluated at 2.21×10^{12} VP/ml obtained by the $OD_{260\,nm}$ method. Five different concentrations were prepared ranging from 1×10^{11} VP/ml to 1×10^{12} VP/ml in duplicate. The mean peak area obtained from the absorbance profile at 260 nm was then plotted against the reovirus concentrations, which gave a linear relationship with a correlation coefficient (R^2) of 0.99901, slope of 1.20×10^{-6} and *Y*-intercept of -6.27×10^4. The standard errors were 15218 for mean peak area, 1.32×10^4 for the Y-intercept and 2.18×10^{-8} for the slope. Unknown reovirus samples were quantified using the linear regression equation as long as the sample fell within the range of the standard curve. Generally, a standard curve was generated only once each time a new column was prepared and the same curve was used for quantification during the entire lifetime of the column. To ensure the validity of the curve, an assay control (Reolysin® standard diluted to a final concentration of 7×10^{11} VP/ml) was always analyzed prior to any sample analyses. In a total number of 36 analyses of the control, the actual VP/ml obtained using the same curve for a period of 3 months using one column fell within ±10% of 7×10^{11} VP/ml (data not shown). The same standard curve can therefore be used day-to-day, week-to-week during the entire lifetime of the column. When the actual concentration of the control shifted out of this range, a new column was prepared. With regards to reproducibility of the standard curve, so far we have used a total of three columns since the validation of this assay [5] and we have found that the slopes obtained were quite similar with a mean of 1.22×10^{-6} and 2% R.S.D.

3.6. Production kinetic monitoring

The developed HPLC method was used to monitor the reovirus concentration in culture supernatants collected at different hpi during reovirus productions at the 20 L, 40 L and 100 L scale, respectively. Representative profiles of the culture supernatants collected at 24 hpi, 48 hpi, 71 hpi and 95 hpi are shown in Fig. 5A–D, respectively. The reovirus peak in all the profiles is indicated by an arrow. Table 1 shows the viral particle concentration results obtained from a typical 20 L, 40 L and 100 L production. For the 20 L production, the VP/ml obtained for the 24 hpi sample was below the detection limit of the assay (1.10×10^{10} VP/ml at 50 μl sample volume injection), whereas for the 54 hpi, 72 hpi and 96 hpi samples were 2.94×10^{11} VP/ml, 3.94×10^{11} VP/ml and 7.02×10^{11} VP/ml, respectively. For the 40 L productions, the VP/ml obtained for the 24 hpi sample was also below the detection limit of the assay whereas for the 50 hpi, 65 hpi and 90 hpi samples were 4.97×10^{11} VP/ml, 8.39×10^{11} VP/ml and 1.01×10^{12} VP/ml, respectively. For the 100 L production, the VP/ml obtained were 4.28×10^{10} VP/ml, 4.37×10^{11} VP/ml, 4.75×10^{11} VP/ml and 3.47×10^{11} VP/ml for the 24 hpi, 48 hpi, 71 hpi and 95 hpi, respectively. In the case of the 20 L and 40 L productions, it was observed that as the production progressed, the viral particle accumulation in the culture supernatants collected at different hpi was inversely proportional to the percentage in cell viability of the producer cell (data not shown). This observation supported the process of reovirus release in the supernatant due to cell lysis during production. This trend however was not entirely demonstrated with the 100 L production as the VP/ml decreased at 95 hpi. Noticeable as well was the constant viral particle accumulation at 71 hpi. Several possible causes, such as cell density at infection or bioreactor configuration might explain this difference in results from the other two runs. To further confirm these assumptions, future studies, out of the scope of this work, need to be performed.

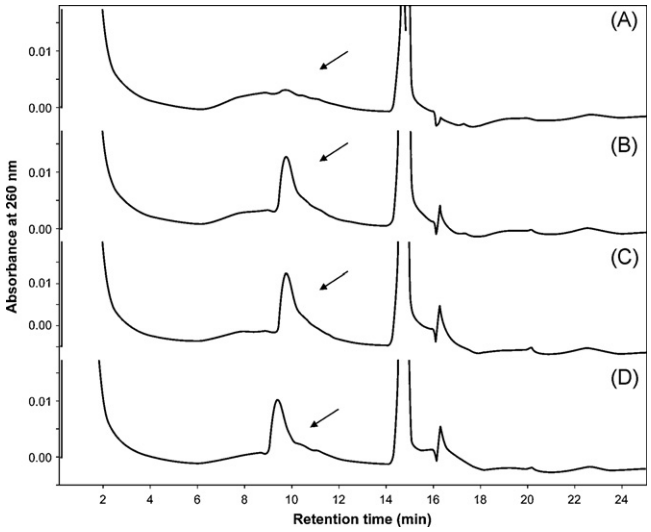

Fig. 5. HPLC profiles of reovirus in culture supernatant collected at different hours post-infection (hpi). Figures (A–D) are samples at 24 hpi, 48 hpi, 71 hpi and 95 hpi, respectively during the production of Reolysin® at the 20 L scale. The virus peak is indicated by an arrow in all the profiles.

3.7. Downstream process monitoring of reovirus particles

The monitoring of reovirus particles was performed using the developed method during the optimization of conditions in the current downstream process to manufacture Reolysin®.

During this study, three 5 L runs were performed and all critical and relevant samples at each step of the process were analysed. Unless otherwise specified all samples were always analysed fresh without prior storage at 4 °C or −80 °C. Run #1 was a replication of the current process with a minor change in the concentration/diafiltration and IEC steps. Run #2 was the incorporation of major changes in the process based on the results obtained from Run #1. Run #3 was a refinement of the process with minor changes based on the results obtained from Run #2. The detailed changes in the process will not be discussed in this paper. A representative profile of the critical samples at each step of the downstream process

Table 1
Reovirus particle concentration (VP/ml) in culture supernatant collected at different hours post-infection (hpi) during Reolysin® production at the 20 L, 40 L and 100 L scale

20 L		40 L		100 L	
Sample (hpi)	VP/ml ($\times 10^{-11}$)	Sample (hpi)	VP/ml ($\times 10^{-11}$)	Sample (hpi)	VP/ml ($\times 10^{-11}$)
24	<0.11[a]	18	<0.11[a]	24	0.43
54	2.94	50	4.97	48	4.37
72	3.94	65	8.39	71	4.75
96	7.02	90	10.10	95	3.47

[a] 1.10×10^{10} VP/ml = quantification limit of the assay at 50 μl sample volume injection.

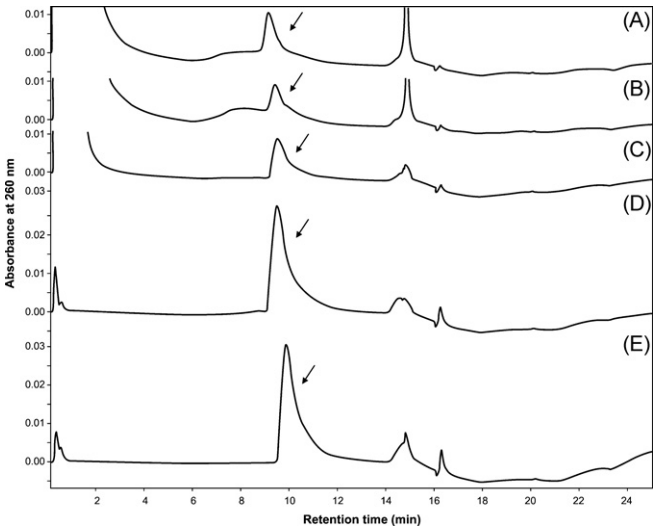

Fig. 6. HPLC profiles of major samples at each step of the downstream process to purify Reolysin®. Figure (A) is the non-clarified virus lysate which was also the starting material, figure (B) is the clarified virus lysate, figure (C) is the concentrated/diafiltered virus, figure (D) is the ion-exchange chromatography eluted virus and figure (E) is the gel permeation chromatography purified virus filtered through 0.22 μm membrane which is also the Reolysin®. The virus peak is indicated by an arrow in all the profiles. The samples in (A)–(C) were analysed with no dilution while the samples in (D) and (E) were analysed at a dilution of 1:5 in 50 mM HEPES, pH 7.10.

are shown in Fig. 6A–E for the virus lysate, the clarified virus lysate, the concentrated and diafiltered virus, the IEX eluted virus, and the final purified product (Reolysin®) which is also the GPC purified virus filtered through 0.22 μm membrane, respectively. The virus lysate, non-clarified and clarified, concentrated and diafiltered virus were analyzed without sample dilution whereas the IEX eluted virus and the final purified product were analyzed with 1:5 dilution. An arrow indicates the virus in all the profiles. Worth mentioning is that during the optimization of the DSP conditions, relevant samples at every step of the process thought to have an impact on particle recovery were routinely analyzed. These samples for example are the virus retentate during the diafiltration step and the IEX column flowthrough just to name a few. Table 2A shows the summary of particle concentration and total recovery at each step of the process during the optimization of the conditions. An improvement in the total VP recovery from 11% to 31% and 48% with run #1, run #2 and run #3 was obtained. Similarly with the IEX step, the total virus recovery improved from 33% with run #1 to 82% and 93% with runs #2 and 3, respectively. The virus infectious titer of the final purified product also showed an improved TCID50 recovery from 12% in run #1 to 30% and 35% in runs #2 and run #3, respectively (Table 2B). It is important to mention here that the TCID50 assay which is known to be inherently variable had a 61% inter-variability and from 0% to 80% intra-assay variability ($N = 34$). Despite the high variabilities, the TCID50 recovery was shown to have improved after the major changes in the process were implemented.

The optimized process was further scaled up to 20 L, 40 L and 100 L and the developed method was routinely used for the quantification of viral particles and total virus recovery at each step of the process. The summary of results is shown in Table 3. The process was reproducible with final VP recoveries of 41%, 41% and 36% for the 20 L, 40 L and 100 L with no difference in the final viral concentrations obtained at 9.85×10^{11} VP/ml, 1.86×10^{12} VP/ml and 1.24×10^{12} VP/ml, respectively.

Table 2
Results summary of reovirus particle concentration (VP/ml) and total virus recovery during the optimization of the downstream process at the 5 L scale for the manufacturing of Reolysin®

Sample ID	VP/ml ($\times 10^{-11}$)			Total virus recovery (%)		
	Run #1	Run #2	Run #3	Run #1	Run #2	Run #3
A:						
Non-clarified virus lysate	4.29	2.27	2.76	100	100	100
Clarified virus lysate	3.78	2.33	2.88	90	101	98
Concentrated/diafiltered virus	8.69	5.27	11.60	62	106	102
Ion exchange eluted virus	36.0	25.2	24.0	33	82	93
Final purified product[a]	6.79	6.70	7.78	11	31	48
Sample ID	TCID50/ml ($\times 10^{-10}$)			Total Virus Recovery (%)		
	Run #1	Run #2	Run #3	Run #1	Run #2	Run #3
B:						
Non-clarified virus lysate	7.04 ± 2.06	3.82 ± 0.91	4.91 ± 3.44	100	100	100
Clarified virus lysate	3.08 ± 0.48	4.48 ± 1.64	2.60 ± 0.62	54	116	51
Concentrated/diafiltered virus	9.48 ± 0.67	3.98 ± 0.91	4.40 ± 1.53	42	48	46
Ion exchange eluted virus	52.4 ± 7.71	30.4 ± 26.0	NA	38	59	NA
Final purified product[a]	11.7 ± 1.52	11.0 ± 1.90	10.5 ± 2.51	12	30	35

TCID50/ml results are mean ± standard deviation of triplicate analyses. NA: not applicable. All wells were contaminated.
[a] Reolysin® = gel permeation chromatography purified and filtered through 0.22 μm.

Table 3
Summary of reovirus particle concentration (VP/ml) and total virus recovery during the downstream process scale-up at the 20 L, 40 L and 100 L of Reolysin® manufacturing

Sample ID	VP/ml ($\times 10^{-12}$)			Total virus recovery (%)		
	20 L	40 L	100 L	20 L	40 L	100 L
Non-clarified virus lysate	0.72	0.88	0.34	100	100	100
Clarified virus lysate	0.70	0.66	0.24	95	72	96
Concentrated/Diafiltered virus	1.03	0.92	0.42	106	77	82
Ion exchange chromatography purified virus	2.93	3.63	2.42	79	55	81
Final purified product[a]	0.99	1.86	1.24	41	41	36

[a] Reolysin® = gel permeation chromatography purified and filtered through 0.22 μm.

4. Conclusion

This HPLC method is an important tool to efficiently optimize and scale-up the downstream process in the manufacture of Reolysin®. Because it is fast, specific and reliable, the quantification of particle concentration and total virus recovery at every step of the process facilitated the optimization of the process that resulted in an improved recovery both in total and infectious particles. With this HPLC method, results were obtained in a matter of minutes. The method was instrumental for the quick and wise decision-making during the development of the process that otherwise would not have been possible by just relying on the titration assay that takes 14 days before any results could be obtained. Therefore, the scale-up of the process to the 20 L, 40 L and 100 L scale was successfully achieved with reproducible total virus recoveries. The developed method is suitable in the analysis of culture supernatants, in-process samples and final purified products and is an ideal monitoring tool during the development and scale-up of the Reolysin® production process. This method has been validated according to regulatory guidelines and has been transferred into current good manufacturing practices (cGMP) facilities for the production of clinical grade material.

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References

[1] M.C. Coffey, J.E. Strong, P.A. Forsyth, P.W. Lee, Science 282 (1998) 1332–1334.
[2] J. Stoeckel, J.G. Hay, Curr. Opin. Mol. Ther. 8 (2006) 249–260.
[3] http://www.oncolyticsbiotech.com.
[4] R.E. Smith, H.J. Zweerink, W.K. Joklik, Virology 39 (1969) 791–810.
[5] P.S. Chahal, J. Transfiguracion, A. Bernier, R. Voyer, M. Coffey, A. Kamen, J. Pharm. Biomed. Anal. 45 (2007) 417–421.
[6] R. Alain, F. Nadon, C. Seguin, P. Payment, M. Trudel, J. Virol. Methods 16 (1987) 209–216.
[7] L.J. Reed, H. Muench, Am. J. Hygiene 27 (1938) 493–497.
[8] K.M. Coombs, Virology 234 (1998) 218–228.
[9] J. Jiang, K.M. Coombs, J. Virol. Methods 128 (2005) 88–92.
[10] R. Floyd, D.G. Sharp, Appl. Environ. Microbiol. 38 (1979) 395–401.
[11] P.J. Gomatos, I. Tamm, Proc. Natl. Acad. Sci. 49 (1963) 707–714.