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Validation of a high-performance liquid chromatographic assay for the quantification of Reovirus particles type 3

Parminder Singh Chahal [a], Julia Transfiguracion [a], Alice Bernier [a], Robert Voyer [a], Matt Coffey [b], Amine Kamen [a,*]

[a] *Animal Cell Technology, Bioprocess Sector, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montreal, Quebec H4P2R2, Canada*
[b] *Oncolytics Biotech Incorporated, 210, 1167 Kensington Cr NW, Calgary, Alberta T2N 1X7, Canada*

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Abstract

An anion exchange high-performance liquid chromatography (HPLC) method for the quantification of human Reovirus type 3 particles was validated according to the performance criteria of precision, specificity, linearity of calibration and working range, limits of detection and quantification, accuracy and recovery. Samples taken at various stages of Reovirus purification were used for the validation of the method. The method was specific for Reovirus which eluted around 9.8 min without interference from any other component in the sample. Reovirus can be detected between 0.32E+12 and 2.10E12 VP/mL by the proposed method that has the correlation coefficient of linearity equal to 0.9974 and the slope of linearity equal to 5.74E−07 area units/(VP mL).

Keywords: Reovirus; Oncolytics; HPLC; Quantification; Validation

1. Introduction

Reovirus (Respiratory Enteric Orphan Virus), a benign human virus has shown oncolytic properties [1,2] and is being used currently on clinical trials for therapeutic treatments of various human cancers. To support these activities, a large amount of clinical material is needed requiring the existing manufacturing process to be scaled up. Therefore, to facilitate the scale-up and a rapid success in developing robust processes, a fast, specific and reliable quantification method would be a great asset. Reovirus is a non-enveloped icosahedral virus, 80 nm in diameter with a genome of 10 segments of double-stranded RNA. Currently, the method used to determine the Reovirus concentration is the $TCID_{50}$ that takes 7–14 days for analysis. An anion exchange HPLC method using a UNO-Q polishing column (Bio-Rad Laboratories) was therefore developed (to be published separately) for the rapid in-process and final product quantification of Reovirus particles. This paper presents the validation data of this method according to regulatory guidelines.

2. Materials and methods

The method was validated according to the guidelines suggested by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), especially: ICH-Q2B [3], ICH-Q2(R1) [4] and US FDA [5]; and the publications of Szepesi et al. [6] and Ritter et al. [7]. The method was validated with samples obtained at various steps of the purification process representing crude, semi-purified and purified forms of Reovirus solutions.

2.1. HPLC system

An HPLC System (Alliance, Waters Ltd., Milford, MA) equipped with a 2690 separations system, a 996 photodiode array detector (PDA), 717 Plus autosampler, Waters 600 controller, and Waters on-line degasser was used in this study. Millennium32 software was used for data acquisition and peak integration. A UNO-Q polishing column (4.6 mm × 10 mm)

* Corresponding author. Fax: +1 514 496 6785.
E-mail address: amine.kamen@cnrc-nrc.gc.ca (A. Kamen).

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from Bio-Rad Laboratories was used to isolate the Reovirus peak from the rest of the components present in the sample. The column was equilibrated with buffer A (0.25 M HEPES, pH 7.1):buffer B (2 M NaCl):water (purified using a Milli-Q® water purifying system) mixture with 20.0:7.5:72.5 ratio. All buffers were filtered through 0.45 μm surfactant-free HATF membrane (Millipore). After equilibration of the column, buffer blanks in triplicates (50 mM HEPES, pH 7.1) were injected to ensure a flat baseline. After 10 consecutive injections of the samples, at least two buffer blanks were run to avoid accumulation of material on the column. Sample injection volume was 25 μL unless otherwise mentioned. Samples or standards were diluted in buffer (50 mM HEPES, pH 7.1) whenever necessary. After 10 min of column wash with 150 mM NaCl at 1.0 mL/min, a gradient from 150 to 340 mM NaCl for 6 min was applied. The column was then washed by 2 M NaCl for 4 min at 0.5 mL/min and brought to 0 M NaCl for 3 min at 1 mL/min before the next injection. The chromatogram was obtained in terms of absorbance at 260 nm. The peak area was used for calibration and concentration determination.

It should be mentioned that the method included a cleaning and regeneration steps for each injection run, however, it was important to replace the guard column frequently. Build up of pressure in the HPLC system was used as a guide to replace the column or column guard. Although the results in this manuscript were obtained from a single lot, this method has been successfully tested on three different lots of BioRad columns.

2.2. Reovirus standard preparation

Virus quantification was performed using an in-house reference standard that was purified according to Oncolytics Biotech Inc. manufacturing process of Reovirus (Reolysin®). The process included four major steps: (1) cell lysis, (2) ultrafiltration/diafiltration (UF/DF), (3) purification by ion-exchange chromatography (IEC) and (4) polishing by gel permeation chromatography followed by 0.2 μm filtration (final product). The concentration of the purified virus was evaluated at 2.10E12 VP/mL and was obtained by measuring the absorbance at 260 nm according to the previously published methods [8,9]. This method can be applied only to the purified material because it is empirically derived from the absorbance at 260 nm related to the components of virus and their percent distribution. Therefore, the crude or semi-pure virus preparations will be over estimated. The samples taken at various stages of the process were frozen at −80 °C.

2.3. Experimental procedure

All samples were filtered through 0.2 μm, Acrodisc with Supor membrane (Pall Life Sciences) prior to the analysis by HPLC. A 25 or a 13 mm diameter filter was used for sample volumes between 1 and 2 mL or between 200 and 300 μL, respectively.

2.3.1. Precision

Six repeated injections (intra-day) of four samples: (A) cell lysate, (B) UF/DF product, (C) IEC eluted virus peak and (D) final product, were injected for five consecutive days (inter-day) to measure the repeatability. The means of the peak area and the retention time of the six injections were determined and the overall means for five consecutive days were calculated along with the standard deviations. The results are reported as percent relative standard deviation (R.S.D.) in Table 1.

2.3.2. Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD), was calculated from the concentration equivalent to 3 standard deviation (S.D.) above the mean peak area at 9.8 min of 15 samples. The limit of quantification (LOQ) was calculated from the 10 S.D.

2.3.3. Linearity of calibration and working range

Purified Reovirus in concentration range from 0.10E+12 to 2.12E+12 VP/mL was injected in HPLC. The linearity was determined by plotting peak area as a function of Reovirus concentration. Slope and *y*-intercept were determined. Percent difference from the expected value was plotted against the expected value of Reovirus concentration and where the curve of the plot started to deviate from an arbitrary level of 20%, a lower or upper range of linearity was set.

2.3.4. Specificity of Reovirus peak

A HEPES buffer blank, a purified Reovirus standard, uninfected cell lysate, uninfected cell lysate spiked with Reovirus standard, infected cell lysate, and an infected cell lysate spiked with Reovirus standard, all in HEPES buffer were visually inspected for the presence of substances which might have co-eluted in the Reovirus peak.

2.3.5. Recovery and accuracy

The closeness of the results to the true value representing the accuracy of the method was tested by triplicate injections of equivalent amounts of Reovirus used for spiking in HEPES buffer, cell lysate and final product samples. Further, cell lysate and final product were spiked with 0.30E+12 Reovirus particles per milliliter. The percent recovery of the spiked Reovirus was then calculated.

3. Results and discussion

3.1. Specificity

No peak was found in HEPES buffer that could interfere with purified Reovirus standard peak (Fig. 1a and b). It was observed that an elevated baseline “bump” around the elution time of the virus elution was present with the non-spiked non-virus infected cell lysate (Fig. 1c and d). The bump was a result of buffer and gradient generation, because all the samples were diluted in the same buffer (50 mM HEPES, pH 7.1). There were buffer blank injections before each sample injection of Fig. 1, and no carryover peaks were observed by injecting sequential buffer blanks after the injection of sample. This “bump” however showed no interference, because

Table 1
Intra-day and inter-day assay

	Intra-day					Inter-day
	Day 1	Day 2	Day 3	Day 4	Day 5	
(A) Cell lysate						
Peak area						
Mean	212,850	209,314	200,114	200,373	206,089	205,748
S.D.	3966	6903	8160	7749	12,058	5566
R.S.D.	1.86	3.3	4.08	3.87	5.85	2.71
N	6	6	6	6	6	6
Retention time						
Mean	9.566	9.514	9.555	9.479	9.407	9.504
S.D.	0.022	0.027	0.024	0.022	0.088	0.064
R.S.D.	0.23	0.29	0.25	0.23	0.94	0.68
N	6	6	6	6	6	6
(B) UF/DF product						
Peak area						
Mean	169,916	163,112	149,604	143,057	130,942	151,326
S.D.	7404	5361	4401	5608	4392	15,583
R.S.D.	4.36	3.29	2.94	3.92	3.35	10.30
N	6	6	6	6	6	6
Retention time						
Mean	9.66	9.488	9.51	9.504	9.363	9.505
S.D.	0.023	0.013	0.019	0.037	0.046	0.105
R.S.D.	0.24	0.14	0.2	0.39	0.49	1.11
N	6	6	6	6	6	6
(C) IEC virus eluted peak						
Peak area						
Mean	177,498	166,994	137,938	142,205	146,012	154,129
S.D.	2168	1471	2750	3429	1946	17,189
R.S.D.	1.22	0.88	1.99	2.41	1.33	11.15
N	6	6	6	6	6	6
Retention time						
Mean	9.681	9.572	9.543	9.534	9.47	9.560
S.D.	0.018	0.041	0.036	0.014	0.05	0.077
R.S.D.	0.18	0.43	0.37	0.15	0.53	0.81
N	6	6	6	6	6	6
(D) Final product						
Peak area						
Mean	110,080	104,605	100,700	105,917	107,100	105,680
S.D.	9204	8275	3603	6465	2869	3442
R.S.D.	8.36	7.91	3.58	6.10	2.68	3.26
N	6	6	6	6	6	6
Retention time						
Mean	9.876	9.387	9.522	9.950	9.956	9.74
S.D.	0.009	0.036	0.033	0.019	0.021	0.266
R.S.D.	0.09	0.38	0.35	0.19	0.21	2.77
N	6	6	6	6	6	6

Sample injection was 25 μL.

the spiked peak appeared on top of the "bump". Similarly, in Fig. 1e and f, it was observed that a small peak of Reovirus for the virus-infected lysate increased in height without any other interference when spiked with purified Reovirus. These comparative graphs revealed that the Reovirus peak was an independent peak that elutes at about 9.8 min with no apparent interfering or co-eluting peaks with similar retention times.

3.2. Precision

Table 1 showed a good repeatability for the peak area and retention time for intra-day precision for all samples from days 1 to 5. The R.S.D. was less than 10% for the peak area and less than 1% for the retention time. The inter-day precision was less than 15% for peak area and less than 3% for the peak retention time for all the samples.

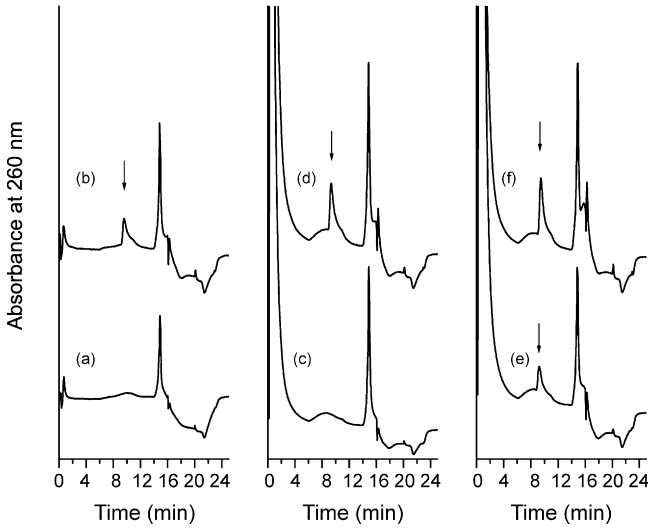

Fig. 1. Specificity of Reovirus peak in HPLC profiles: (a) buffer blank; (b) buffer blank spiked with 0.30E+12 VP/mL purified Reovirus; (c) cell lysate from un-infected cells; (d) cell lysate from un-infected cells spiked with 0.30E+12 VP/mL Reovirus; (e) cell lysate from infected cells; (f) cell lysate from infected cells spiked with 0.30E+12 VP/mL Reovirus. The downward arrows point to the Reovirus peak.

3.3. Limit of detection (LOD) and limit of quantification (LOQ)

The mean and S.D. for 15 buffer blanks were 10,546 and 2044, respectively. Therefore, LOD and LOQ were calculated to be 16,679 and 30,990 area units, which corresponded to 2.14E+11 and 2.39E+11 VP/mL, respectively.

3.4. Linearity of calibration and working range

Fig. 2 showed the calibration curve along with the percent difference from the expected value for all the concentrations tested.

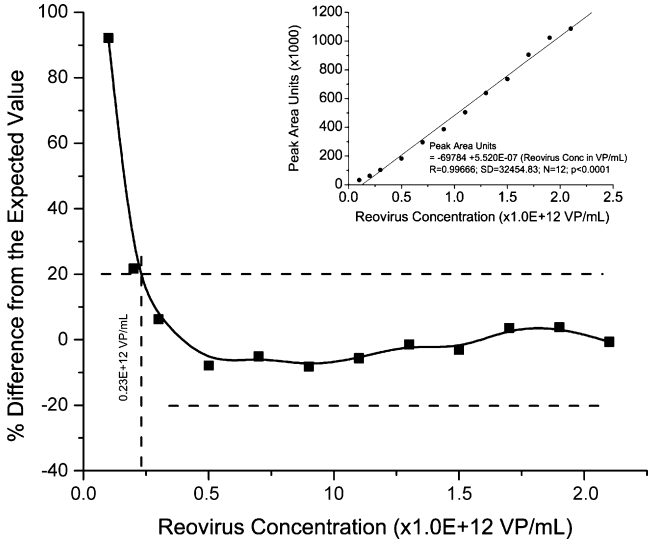

Fig. 2. Percent difference from the expected value predicted by linearity curve for data points plotted between 0.10E+12 and 2.10E12 VP/mL. Inset: calibration plot for data points between 0.10E+12 and 2.10E12 VP/mL.

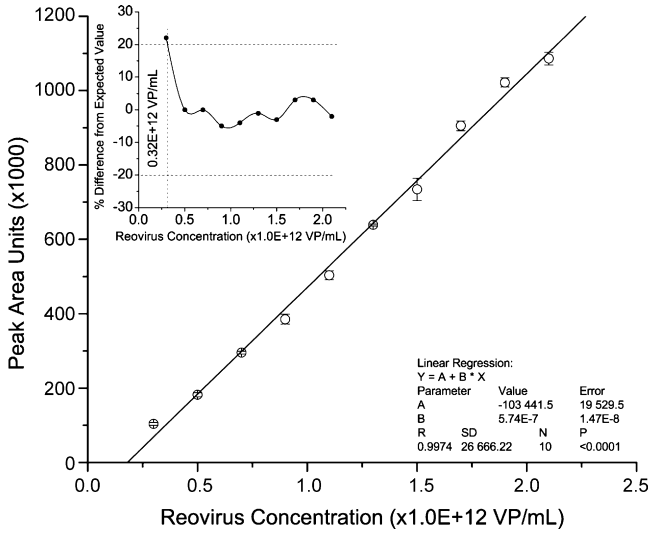

Fig. 3. Calibration plot for data points within ±20% from the expected value defined by Fig. 2. Inset: percent difference from the expected value predicted by linear curve for data points plotted between 0.30E+12 and 2.10E12 VP/mL.

All triplicate injections of each concentration gave an R.S.D. of less than 5%. The range of linearity was thus defined between 0.23E+12 and 2.10E12 VP/mL (Fig. 2). Upper limit of linearity was not revealed by this procedure because the maximum concentration of Reovirus available was only 2.10E12 VP/mL. Higher concentrations of Reovirus had tendency to form aggregates. A new linear calibration curve (Fig. 3) was obtained by using newly defined range. This calibration had a correlation coefficient of 0.9974 and a slope of 5.74E−07 area units/(VP mL). The calibration curve had a S.D. of 26,666 with p value <0.0001, suggesting that it was a good correlation. The percent difference of the expected and the predicted value was again calculated with the new calibration parameters and showed that 0.30E+12 VP/mL concentration produced more than 20% error (inset of Fig. 3). Assuming a ±20% acceptance criteria for biological compounds such as viruses, the linear working range for determining Reovirus concentration was set to be between 0.32E+12 and 2.10E12 VP/mL. The y-intercept had ±95% confidence intervals of −142,109 and −64,772 area units, which were significantly different from 0. Therefore, this curve could not be forced through 0. With 95% confidence level, the slope between 5.45E−07 and 6.03E−07 area units/(VP mL) should be acceptable.

Since the linear fit did not pass through 0, at least two-point calibration preferably three-point calibration must be done prior to running the samples. For three point calibration, one can choose an average concentration of virus that is expected in the process and the other two concentrations could be ±30% of the average concentration expected in the process, provided all three concentrations fall within the range of linearity established above. In our case, for generation of calibration curve, we use 3.50E11, 5.00E11 and 6.50E11 VP/mL.

3.5. Recovery and accuracy

The amount of Reovirus that was spiked gave a final concentration of 0.31E+12 VP/mL (average of triplicate samples) when

Table 2
Recovery of spiked purified Reovirus at a concentration of 0.30E+12 VP/mL

	Reovirus 0.30E+12 VP/mL (peak area)	Cell lysate (peak area)	Cell lysate spiked with Reovirus (peak area)	Recovery of Reovirus peak area	Percent recovery of Reovirus
A. Cell lysate sample					
Mean	69,176 = 0.31E+12 VP/mL	55,814	125,032	69,218 = 0.31E+12 VP/mL	100
S.D.	262	2 711	9 057		
R.S.D.	0.38	4.86	7.24		
N	3	3	3		

	Final product (peak area)	Final product spiked with Reovirus (peak area)	Recovery of Reovirus peak area	Percent recovery of Reovirus
B. Final product sample				
Mean	26,949	87,468	60,519 = 0.29E+12 VP/mL	87
S.D.	979	988		
R.S.D.	3.63	1.13		
N	3	3		

the Reovirus standard was diluted in HEPES buffer (Table 2). The percent recoveries from spiked samples of cell lysates and final product were 100 and 87%, respectively. This corresponded to 0.31E+12 VP/mL and 0.29E+12 VP/mL for the cell lysate and final product, respectively. These recoveries were not significantly different from each other at 95% confidence interval. At 95% confidence level, the range of expected concentration of 0.30E+12 VP/mL of Reovirus was between 0.285E+12 VP/mL and 0.315E+12 VP/mL. Therefore, accuracy of this method is considered to be good for a purified sample (final product) as well as for the crude (non-purified) sample (cell lysate).

4. Conclusions

The anion exchange—HPLC method using UNO-Q polishing column for the quantification of total Reovirus particles has satisfied all the criteria suggested by ICH guidelines and therefore can be used as a validated method for determining Reovirus particle concentration during the purification process. This method has been now implemented in our laboratory to determine Reovirus concentration at various stages of Reovirus purification process and has been transferred to a cGMP site to support manufacturing of clinical lots.

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