A reversed phase HPLC method for the quantification of HIV gp145 glycoprotein levels from cell culture supernatants

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A B S T R A C T

A reversed phase high performance liquid chromatography (RP-HPLC) method was developed for the quantitative determination of recombinant HIV-1 gp145 produced in CHO-K1 cells, as measured directly from culture supernatants. Samples were diluted in 50% D-PBS and 50% PowerCHO-2 (PC2) spent medium, and resolved on a Zorbax 300SB-C8 Rapid Resolution (2.1 × 50 mm, 3.5 μm) column, fitted with a C8 guard column (Zorbax 300SB-C8, 2.1 × 12.5 mm, 5 μm), using 0.1% TFA and 2% n-propanol in LC-MS water as mobile phase A and 0.1% TFA, 70% isopropanol, and 20% acetonitrile in LC-MS water as mobile phase B. The column temperature was 80 °C, the flow rate was 0.4 mL/min and the absorbance was monitored at 280 nm. The procedures and capabilities of the method were evaluated against the criteria for linearity, limit of detection (LOD), accuracy, repeatability, and robustness as defined by the International Conference on Harmonization (ICH) 2005 Q2(R1) guidelines. Two different variants of the HIV-1 envelope protein (Env), CO6980v0c22 gp145 and SF162 gp140, were analyzed and their retention times were found to be different. The method showed good linearity (R² = 0.9996), a lower LOD of 2.4 μg/mL, and an average recovery of 101%. The analysis includes measurements of accuracy, inter-user precision, and robustness. Overall, we present a RP-HPLC method that could be applied for the quantitation of cell culture titers for this and other variants of HIV Env following ICH guidelines.

1. Introduction

The envelope (Env) glycoprotein that spans the membrane of the human immunodeficiency virus (HIV) and mediates viral infection has been a common template for the design of numerous vaccine candidates [1–9]. In HIV-infected cells, Env is naturally made as a membrane-spanning gp160 glycoprotein that is cleaved into two glycoprotein fragments: a trimeric gp41 and its monomeric binding partner gp120. The monomeric gp120 was used as the boost immunogen in the RV144 clinical trial in Thailand; the only vaccine trial that has so far resulted in significant HIV protection with an efficacy level of 31.2% [10]. The gp120 monomer is still under evaluation in clinical trials in Thailand and South Africa [11–13]. Although some of the early results obtained with the gp120 monomer as a boost immunogen were promising, the development of a globally protective immunogen will require proteins that elicit a more durable and targeted response.

The search for Env-based vaccines of higher efficacy and breadth has led some researchers to look beyond monomeric gp120. More recent immunogen designs have aimed to preserve the trimeric structure of the native Env spike, by including portions of trimer-forming gp41 covalently linked to the otherwise monomeric gp120 [6–8,14]. In this new category of trimeric Env immunogens there are uncleaved trimers (due to a mutated cleavage site), native trimers (gp120:gp41 complexes held together by an engineered disulfide bond), and several genetically fused
con structs (gp120:gp41 held together by a flexible peptide linker). Many of these new trimeric Env constructs, like their monomeric predecessors, are made in mammalian cell expression systems such as CHO and HEK-293, both of which are known to decorate the protein with a native-like glycosylation pattern [15–20]. However, the typical yields of Env-based proteins made in these tested and tested cellular hosts is about Env production directly in the supernatants of cultured cells. To date, optimization of upstream processes [18].

µg/mL) were diluted in 50% CHO-K1 spent medium (final concentration 100 times lower than the yields for other glycoproteins of pharma...

Fig. 1. RP-HPLC method optimization. Representative chromatograms obtained from three injections of the HIV-1 CO6980v0c22 gp145 RM (100 µg/mL) into a Zorbax 300SB-C8 rapid resolution RP-HPLC Column (2.1 × 50 mm, 3.5 µm) mounted on an Agilent BioInert Infinity II 1260 System with different sample preparation and elution gradient schemes. RT, retention time and TF, tail factor. (A) Condition A. The sample was mixed as follows: 40% D-PBS, 40% Mobile phase A, and 20% CHO-K1 spent medium. The elution gradient used was (time/%B): 0/30, 3.5/30, 4/65, 4.5/65, 5.5/75, 6/75, 6.5/80, 7/95, 12/95 and 12.5/35, with a post time of 5 min. (B) Condition B. The sample was prepared as follows: 50% D-PBS and 50% CHO-K1 spent medium. The elution gradient used was (time/%B): 0/30, 2/30, 3.5/65, 4/70, 5/75, 6.5/95, 9.5/95, and 10/30, with a post time of 5 min.

2.3. Chromatographic conditions

Chromatographic separation was performed on an Agilent Technologies (Santa Clara, CA, USA) 1260 Infinity II BioInert HPLC quaternary pump system equipped with a diode array detector (DAD VL-). A reverse-phase C8 column (Zorbax 300SB-C8 Rapid Resolution 2.1 × 50 mm, 3.5 µm) and C8 guard column (Zorbax 300SB-C8, 2.1 × 12.5 mm, 5 µm) were used. The following gradient elution was used (time/%B): 0/30, 2/30, 3.5/65, 4/70, 5/75, 6.5/95, 9.5/95, and 10/30, with a post time of 5 min. The following HPLC running conditions were used: injection volume was 40 µL, flow rate was maintained at 0.4 mL/min, detection was performed at 280 nm (with a reference wavelength set at 320 nm) and column temperature was set to 80 °C. To reduce the carry over, 100 µL of D-PBS 1X was injected between sample or standard injections.

2.4. Evaluation of the RP-HPLC method against ICH guidelines

The RP-HPLC method for the quantitation of CO6980v0c22 gp145 was evaluated against the current criteria for linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, repeatability, and robustness of the ICH guidelines Q2 (R1) (ICH Guidelines, 2005). The LOD and LOQ were calculated using the following equations, respectively, LOD = 3.3 s/S and LOQ = 10 s/S, where s is the standard deviation of intercept and S is the slope of the linear regression curve.

2.5. Cell culture and western blotting

Stably transfected CHO-K1 cells expressing CO6980v0c22 gp145 were grown in PowerCHO 2 (Lonza, Basel, Switzerland) medium supplemented with 10 µg/ml puromycin, 4 mM glutaMAX™ and 1% penicillin/streptomycin. A seeding density of 0.8 million cells/mL was used to inoculate a 40 L vessel controlled by a Finesse G3Lite (ThermoFisher Scientific, Waltham, MA, USA) system with an agitation of 65 rpm, dissolved oxygen 35%, pH 6.8 with CO₂ adjustment, and a reactor temperature of 37 °C. Daily culture samples was collected from the bioreactor for CO6980v0c22 gp145 daily litter analysis. The samples were mixed 1:1 with gel sample buffer and analyzed by SDS-PAGE using 4–20% acrylamide (Invitrogen, ThermoFisher, Waltham, MA, USA). A total of 1.0, 0.5, 0.25, and 0.125 µg was loaded of the reference standards for quantification. For western blot analysis, the gel was obtained from Advanced Bioscience Laboratories (ABL Inc.).

The CO6980v0c22 gp145 reference material (RM) in D-PBS 1X was obtained from CDI Laboratories.
transferred to a nitrocellulose membrane that was subsequently incubated with the broadly neutralizing antibody 4E10 (Polymun Scientific Immunobiologische Forschung GmbH, Klosterneuburg, Austria). The secondary labeling step was performed using an anti-human IgG coupled with alkaline phosphatase (ThermoFisher, Waltham, MA, USA). The resulting bands were visualized on a Chemidoc XRS+ (BioRad, Hercules, CA, USA) and quantified by densitometry using Image Lab™ Software from BioRad. Briefly, a linear standard curve was generated using the gp145 standards of known concentrations (1.0, 0.5, 0.25, and 0.125 µg).

3. Results

3.1. Reversed-phase HPLC method development

Several parameters were tested and optimized during the development of the RP-HPLC method for the determination of gp145 titer from bioreactor supernatants. The most noteworthy changes that resulted in substantial improvements were in sample preparation and gradient elution schemes. Two specific conditions are described here to illustrate how the method was improved (Fig. 1). Condition A involved the dilution of sample in 40% D-PBS, 40% Mobile phase A, and 20% CHO-K1 spent medium with a “fast” gradient (30%B = 65%B in 30 s), whereas Condition B involved the dilution of sample in 50% D-PBS and 50% CHO-K1 spent medium with a slow gradient (30%B = 65%B in 90 s) The results show that the peak shape and recovery were improved as indicated by tail factor (TF) and area (AUP), respectively. The tail factors observed were 2.333 and 1.057 for Condition A and Condition B, respectively. Furthermore, the area under the curve (AUP) for each peak was 738 and 1115 for Conditions A and B respectively. Altogether these results indicated that Condition B was adequate for the titer determination of gp145 supernatants.

3.2. Method specificity

To evaluate the specificity of our analytical method, the spent medium alone (buffer blank) and the spent medium spiked with CO6980v0c22 HIV-1 gp145 were analyzed by RP-HPLC. The standards used were mixed 1:1 with PC2 spent medium to best mimic the sample matrix that we intend to quantify, namely, gp145 supernatants directly from bioreactors. Three consecutive injections of 40 µL were analyzed by RP-HPLC. Peaks arising from spent medium components are only observed between 0 and 2 min (Fig. 2). There is no signal observed from the sample matrix in the region where gp145 elutes, from minute 5 to 6. Thus, the spent medium does not interfere with the measurements of gp145. Since the data show that the gp145 peak is clearly resolved from the spent medium peak, this indicates that the method is specific for gp145 (Fig. 2).

To further challenge our analytical method, gp145 (clade C) and SF162 gp140 (clade B) were analyzed by RP-HPLC. Retention times of 5.385 min and 5.489 min were observed for CO6980v0c22 gp145 and SF162 gp140, respectively (Fig. 2B, Table 1). This data show that our RP-HPLC method can discriminate between these two very similar HIV-1 Env immunogens: CO6980v0c22 gp145 and SF162 gp140.

3.3. Linearity and range

A series of CO6980v0c22 HIV-1 gp145 standard samples, ranging in concentration from 12.5 to 100 µg/mL, each in 50% D-PBS 1X and 50% PC2 spent medium, was injected sequentially into the HPLC system. The

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Retention time (average, minutes)</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO6980v0c22 gp145</td>
<td>5.385</td>
<td>0.001</td>
<td>0.2</td>
</tr>
<tr>
<td>SF162 gp140</td>
<td>5.489</td>
<td>0.001</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 2

<table>
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<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration Range</td>
<td>12.5–100 µg/mL</td>
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<tr>
<td>Intercept</td>
<td>17.79</td>
</tr>
<tr>
<td>Coefficient of Determination (R²)</td>
<td>0.9996</td>
</tr>
<tr>
<td>Standard Error of Intercept</td>
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<tr>
<td>Standard Deviation of Intercept</td>
<td>7.8</td>
</tr>
<tr>
<td>Limit of Detection (LOD)</td>
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</tr>
<tr>
<td>Limit of Quantitation (LOQ)</td>
<td>7.1 µg/mL</td>
</tr>
</tbody>
</table>

Fig. 2. RP-HPLC method specificity. (A) Representative chromatograms obtained from purified gp145 (60 µg/mL in D-PBS) alone and gp145 spiked into spent medium (60 µg/mL in 50% D-PBS and 50% PC2 spent medium). (B) Representative chromatograms obtained from three (3) injections of the HIV-1 CO6980v0c22 gp145 RM in 50% 1X D-PBS and 50% spent medium (60 µg/mL, black solid line), SF162 gp140 in 50% 1X D-PBS and 50% spent medium (60 µg/mL, black dashed line) and a buffer blank (blue solid line) onto a Zorbax 300SB-C8 rapid resolution RP-HPLC column (2.1 × 50 mm, 3.5 µm) using the Agilent BioInert Infinity II 1260 System. The zoom window highlights the peaks observed for the two HIV-1 Env proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
The area under the peak (AUP) was obtained for each sample and the mean and relative standard deviation (RSD) were calculated for each run. In addition, the slope, Y-intercept, coefficient of determination ($R^2$), LOD and LOQ were determined from the linear regression analysis. Results in Table 2 and Fig. 3 show the linear correlation between AUP and the concentration of CO6980v0c22 gp145 in the sample. The $R^2$ for the calibration curve shown in Fig. 2 was 0.9996 and the average for three independent experiments carried out on different days, is 0.997 with a standard deviation of 0.0002. Additional parameters of the regression equation are shown in Table 2.

### 3.4. Accuracy

The CO6980v0c22 gp145 concentrations of 12.5, 25, 37.5, 50, 75 and 100 µg/mL were used to determine the accuracy of the RP-HPLC method. The percent recovery was 100.56 ± 2.44 with a percent RSD below 2.7% (Table 3). Collectively, these results indicate that the RP-HPLC method described herein is suitable for the quantitation of CO6980v0c22 gp145 directly from bioreactor supernatants.

### 3.5. Repeatability

The repeatability (inter-day variation) was determined from the results from six independent injections of a sample containing 60 µg/mL of CO6980v0c22 gp145 (Table 4). The concentration of 60 µg/mL was chosen because it lies in the middle of the linear range. Results show a %RSD of 0.34 and 0.16 for the inter-day variations for USER #1 and #2, respectively. Moreover, for the intermediate precision, the differences between different users were recorded and evaluated (Table 4). The experiments were carried out on different days and with fresh solvent each day. The overall %RSD for the intermediate precision is 1.16.

### 3.6. Robustness

Method robustness was assessed by testing the following parameters: column temperature, column ageing, guard column lot variations, and stability of the gp145 material. For the column ageing, the RP-HPLC method was carried out after 6 months of use and after 1742 injections. Fresh solvent was used each time. Results show an overall %
RSD below 6 for the CO6980v0c22 gp145 concentrations of 12.5, 25, 37.5, 50, 75, 100 µg/mL (Table 5). The overall percent RSD observed for variations in the guard column lots and column temperature (78 °C, 80 °C, and 82 °C) were 1.14% and 1.11%, respectively (Tables 6 and 7).

Moreover, age effects on mobile phase and test sample analysis shows that mobile phase and CO6980v0c22 gp145 solutions are stable for 4 days at 10 °C with an overall % RSD of 2.73% (Table 8).

3.7. Quantitation of HIV-1 CO6980v0c22 gp145 from bioreactors

To challenge the newly developed RP-HPLC method, actual supernatants from a bioreactor run were analyzed and compared against quantification by Western blot analysis. Samples of supernatant from CHO-K1 cells expressing CO6980v0c22 gp145 were removed daily from a 40 L bioreactor and analyzed by RP-HPLC and western blot (Fig. 4). The concentration estimates obtained by western blot were consistently higher than the RP-HPLC measurements. The concentrations measurements by RP-HPLC were performed in triplicates and the average standard deviation was determined to be less than 4% of the total value. A representative chromatogram of the gp145 protein obtained from cell culture supernatant is shown in Fig. 5.

4. Discussion

The production of protein-based vaccines in bioreactors requires the development of methods for the quantitation of the desired product, that are quick, highly selective, accurate, reproducible, and that require minimal sample processing. The production of HIV Env vaccines mainly employs ELISA for the quantification of products secreted into the cultivation media. While ELISA is inexpensive and relatively easy to
implement in any production setting, the waiting times for ELISA incubations and washes can be long and the variability of the measurement can often be too wide for the method to be considered accurate, depending on the antibodies and standards used [27]. Another method for the detection and quantitation of protein products in crude supernatants is the Western blot analysis, which also depends on the recognition of the protein analyte by a primary antibody. The high variability associated with both ELISA and Western blot may arise from their reliance on the quality and specificity of the antibodies used for detection [28].

Here, we report a RP-HPLC method for the quantification of an HIV-1 CO6980v0c22 gp145 vaccine candidate directly from culture supernatants of CHO-K1 cells that does not rely on antibody binding. This method is specific since the addition of CHO PC2 spent medium does not interfere with the quantification analysis of gp145. Moreover, we observed a peak corresponding to the medium components in the first 2 min of the gradient elution, which may arise from aromatic amino acids, such as tyrosine and tryptophan, but this did not interfere or alter the signal observed for gp145 in the 5–6 min time frame. The method described herein was found to be linear within a broad range of concentrations, with a standard deviation within 3%, and an average recovery of 101%. The method was accurate, with 2.7% variability on consecutive measurements. The method was precise, with an inter-day variability of 0.34% and an inter-user variability of 1.2% for two different users. The method was also found to be robust, as the measurements obtained with a 6-month old column (after 1742 injections) were within 5.4%–1.6% of the measurements obtained with a new column. The use of different guard columns did not greatly affect separation, since the guard column variability was found to be 1.1%. Finally, the same measurement was carried out with a standard that had been left at 10°C for 3 days and the measurements differed by less than 2.7%, providing a first insight into the effects of ageing on this highly glycosylated family of vaccines.

A direct comparison between the RP-HPLC method and Western blot analysis indicates that our method is suitable for the determination, with low variability, of protein concentration in a bioreactor (Fig. 4). Our RP-HPLC method registered the daily increase in the concentration of CO6980v0c22 gp145 in the bioreactor, with a variability of approximately 4% of the total value. The Western blot method, however, consistently gave higher concentration values, a commonly observed phenomenon for which we have no explanation, although the limitations of the Western blot as a quantitative technique have been extensively reported [28].

5. Conclusion

In all, we have presented the development of a RP-HPLC method for the detection and quantification of HIV-1 CO6980v0c22 gp145 in CHO-K1 culture supernatants. This method could be easily adapted for the analysis of other glycoproteins produced in mammalian cell systems. Our method was determined to be accurate, precise, robust, and required a running time of 20 min per sample, substantially shorter than the time needed for an ELISA measurement.

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Declaration of Competing Interest

The authors declare that they have no known competing financial
interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found at https://doi.org/10.1016/j.jchrolab.2021.122562.

References


