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Expediting the chromatographic analysis of COVID-19 antibody therapeutics with ultra-short columns, retention modeling and automated method development

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ARTICLE INFO

Keywords:

Covid-19
Casirivimab
Imdevimab
Ultra-short column
Retention modeling
Automation

ABSTRACT

The COVID-19 pandemic necessitated the emergency use authorization (EUA) of several new therapeutics and vaccines. Several monoclonal antibodies (mAbs) were among those authorized for use, and they have served a purpose to provide passive immunity and to help minimize dangerous secondary effects in at-risk and hospitalized patients infected with SARS-CoV-2. With an EUA submission, scientific data on a drug candidate is often collected near simultaneously alongside drug development. In such a situation, there is little time to allow misguided method development nor time to wait on traditional turnaround times. We have taken this dilemma as a chance to propose new means to expediting the chromatographic characterization of protein therapeutics. To this end, we have combined the use of automated, systematic modeling and ultrashort LC columns to quickly optimize high throughput RP, IEX, HILIC and SEC separations for two COVID-19-related mAbs. The development and verification of these four complementary analytical methods required only 2 days of experimental work. In the end, one chromatographic analysis can be performed with a sub-2 min run time such that it is feasible to comprehensively characterize a COVID-19 mAb cocktail by 4 different profiling techniques within a 1-hour turnaround time.

1. Introduction

Antibodies (Abs) are primary components of the adaptive human immune response. Among these antibodies are serum immunoglobulin G (IgG) proteins that mediate immune protection and are continuously synthesized by B lymphocytes even months after an infection. Each Y-shaped structure of an immunoglobulin G has regions that are highly conserved as well as some that are variable so as to impart antigen-binding capable of targeting specific pathogenic antigens [1]. As a result of this unique property, IgG-type antibodies have been developed many times over for specific therapeutic uses. Immunotherapeutic antibodies and other related products (bispecific antibody, antibody-drug-conjugate, fusion protein) can be used to treat both common and rare diseases. Moreover, the number of antibody based

therapeutics continues to exponentially grow [2].

To address recurring waves of SARS-CoV-2 infections, there has been a call for monoclonal antibodies (mAbs) that can neutralize the SARS-CoV-2 virus and minimize adverse secondary effects in severely symptomatic cases. To facilitate the immediate access to life-saving drugs, the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) issued several Emergency Use Authorizations (EUAs) allowing the temporary use of existing drug products (for an unapproved clinical application) or completely new, experimental drug candidates [3]. Till now, four different IgG-type mAbs were granted EUA status, namely tocilizumab, casirivimab, imdevimab and bebtelovimab. Tocilizumab is a humanized anti-interleukin-6 receptor (IL-6R) monoclonal antibody that acts like a specific inhibitor to neutralize key factors implicated in COVID-19-induced cytokine release syndrome (CRS),

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<https://doi.org/10.1016/j.jpba.2022.115039>

Received 7 July 2022; Received in revised form 7 September 2022; Accepted 8 September 2022

Available online 12 September 2022

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which has been shown to be directly responsible for many associated deaths [4]. At the same time, casirivimab and imdevimab are monoclonal antibodies that bind simultaneously to non-overlapping epitopes on the receptor binding domain of the SARS-CoV-2 spike protein. In turn, they are able to block normal virus entry via the angiotensin converting-enzyme 2 (ACE2) receptor [5]. Bebtelovimab has been authorized recently and showed effectiveness versus omicron variants [6]. However, despite their promising therapeutic potential, monoclonal antibodies – like all cell culture expressed immunoglobulins – are complex molecules with a high degree of heterogeneity. To ensure appropriate safety profiles and reproducible efficacy, these mAbs must be thoroughly characterized and tested.

Ultrahigh-pressure liquid chromatography (UHPLC) separation techniques are well-suited to biopharmaceutical analysis, including mAb purity and identity control measurements. In this sense, the use of reversed phase (RP) and hydrophilic interaction chromatography (HILIC) is paramount, as these techniques can be readily coupled with mass-spectrometry and thereby provide in-depth characterization data. Non-denaturing separation techniques are valuable as well. Ion-exchange chromatography (IEX) provides information on charge heterogeneity and size-exclusion chromatography (SEC) provides a means to distinguish mAb monomers from dimers and higher order aggregates and low molecular weight fragments.

Starting up analytical method development work on new drug candidates can be a difficult, time-intensive process. Even in the case of a standard drug authorization process, challenges in establishing and following through on analytical work can bring on repeat delays in drug development and subsequent release. While pursuing emergency use authorization, scientific data that confirms safety and effectiveness is to be submitted in real-time alongside drug development. This necessitates employing state-of-the-art techniques and powerful software tools in order to minimize method development time and to maximize method throughput. Novel automated screening platforms have already been introduced and demonstrated to greatly reduce time spent in the very initial stages of method development with minimal manual intervention [7–10].

In this sense, computerized retention method modeling plays an important role and can be applied to speed up the entire analytical process by reducing the number of wet-lab experiments and quickly contextualizing complex chromatographic data [11]. Modeling tools typically comprise various design-of-experiment approaches (or so-called inverse methods) and they approach modeling with either a purely statistical approach or by employing chromatographic fundamentals derived retention models, such as the linear solvent strength (LSS), quadratic (Q), adsorption (ADS), mixed-mode (MM) and Neue-Kuss (NK) models. Each of these make an attempt to describe certain regularities seen for retention factors (log-linear, log-log relationship) when observed as a function of mobile phase composition (ϕ). These retention models have been used across all major LC-modes, including RP, HILIC, normal-phase chromatography (NPLC), IEX and hydrophobic interaction chromatography (HIC).

Another interesting modeling principle is the so-called direct approach, which is free from any previous conventions. The direct approach focuses on accurate measurements of column and LC system properties, and it utilizes complex mathematical models to establish universal thermodynamic rules that can be used to produce retention-factor simulations [12]. Despite this being a powerful tool to better understand complex (e.g. mixed-mode) retention mechanisms, it has not yet been widely applied. At present, pharmaceutical analysts have tended to use indirect retention modeling methods because of their practicality. Typically, industry has used indirect retention modeling to perform method optimization, method transfers, stationary phase characterization and robustness quantification studies [13–16]. To streamline a modeling-based analytical quality-by-design (AQBD) workflow, along with others, our team has also proposed generic modeling approaches for monoclonal antibody and

antibody-drug-conjugate separations [17–22].

On to modeling approaches, it is equally important to consider the retention behavior of large proteins and how they tend to be extremely sensitive to the mobile phase composition. For instance, in many types of separations, a large protein will be eluted with only a very minor change in eluent strength. This phenomenon is referred to as “on-off” or “bind and elute” retention mechanism [23]. The prevalence of this effect increases with molecule size (hydrophobic surface) during RPLC and with the number of available charges in IEX. In this regard, the linear solvent strength (LSS) model is frequently applied to describe protein retention. A consequence of “on-off” elution behavior is that only the first segments of a column will actively take part in peak retention and separation [24]. Therefore, very short columns can be used without comprising the separation efficiency.

In this study, it was our goal to quickly optimize high throughput RP, IEX, HILIC and SEC separations of two COVID-19-related mAbs through the combined use of automated, systematic modeling and various 15–20 mm long columns. With our new approach, the development and verification of four complementary analytical methods required only 1–2 days of experimental work. In the end, one chromatographic analysis can be performed with a sub-2 min run time such that it has become feasible to comprehensively characterize a COVID-19 mAb cocktail by 4 different profiling techniques within a 1-hour turnaround time.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (ACN) and methanol (MeOH) were obtained from Fisher Chemical (Reinach, Switzerland). Trifluoroacetic acid (ULC-MS, TFA, $\geq 99.0\%$) was obtained from Biosolve BV (Valkenswaard, the Netherlands). DL-dithiothreitol (DTT, $\geq 99\%$) was obtained from Axon Lab AG (Baden, Switzerland). Sodium chloride (NaCl, $\geq 99.5\%$), potassium chloride (KCl, $\geq 99.5\%$), di-sodium phosphate (Na_2HPO_4 , $\geq 99\%$), monopotassium phosphate (KH_2PO_4 , $\geq 99.5\%$) and 2-(N-morpholino)ethanesulfonic acid monohydrate (MES, $\geq 99.0\%$) were obtained from Sigma-Aldrich (Buchs, Switzerland). 1 M sodium hydroxide solution (NaOH) was purchased from VWR Chemicals (Rosny-sous-Bois, France). FABRICATOR™ IdeS enzyme was purchased from Genovis AB (Lund, Sweden). Type 1 water was obtained from a Milli-Q™ purification system from Millipore (Bedford, MA, USA). Difluoroacetic acid (DFA), BioResolve™ CX pH Concentrate A (pH 5) and BioResolve CX pH Concentrate B (pH 10.2) were obtained from Waters (Milford, MA, USA). Casirivimab and Imdevimab were obtained from their respective manufacturers as European Union pharmaceutical-grade drug products.

2.2. Instrumentation, columns and software

Modeling (method optimization) experiments were performed on an ACQUITY™ UPLC™ H-Class Plus System (Waters) equipped with a quaternary solvent delivery pump, flow-through needle (FTN) sample manager, a fluorescence detector (FLD) and UV (TUV) detector. The system dwell volume and extra-column volume were measured as $V_d = 0.355\text{ mL}$ and $V_{EC} = 11\ \mu\text{L}$, respectively.

For ultrashort column experiments (optimized and transferred methods), an ACQUITY UPLC I-Class System (Waters) was used to reduce extra-column dispersion and gradient delay volume. This UHPLC system was equipped with a binary solvent delivery pump, flow-through needle (FTN) sample manager, and a fluorescence detector (FLD). A system dwell volume of $V_d = 0.110\text{ mL}$ and extra-column volume of $V_{EC} = 7.5\ \mu\text{L}$ were measured. Fluorescence detection was performed using: $\lambda_{ex} = 280\text{ nm}$ and $\lambda_{em} = 360\text{ nm}$, 10 Hz; and UV detection at 280 nm. Instrument control and data acquisition were performed with Empower™ Pro 3 software.

For RPLC analysis the BioResolve RP mAb Polyphenyl Columns (commercial 2.1 mm \times 50 mm and prototype 2.1 mm \times 20 mm, 2.7 μm ,

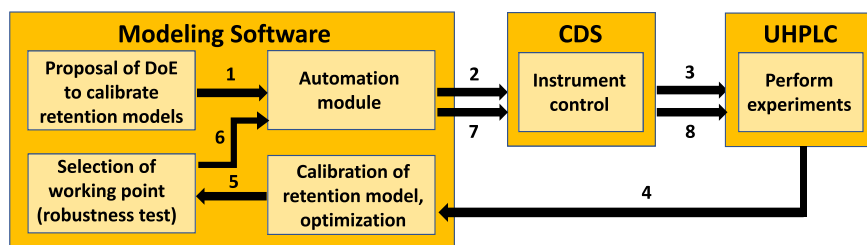


Fig. 1. Schematic view of the automated method optimization process. Modeling software: DryLab, CDS: Empower, Chromatographic system: ACQUITY UPLC. Steps of the procedure: Selection of retention model and experimental design (DoE) and sending variables to automation module (1), transferring model variables to method variables and sending to CDS (2), performing experiments (UHPLC controlled by CDS) (3), transferring measured chromatograms and retention data to the modeling software to calibrate the retention model (4), selection of working point (optimization) (5), transfer optimal conditions to the automation module (6), sending method variables (optimal

condition) to CDS (7) and performing experimental verification of the selected working point (8).

450 Å) were used. HILIC analysis was performed using the Waters ACQUITY UPLC Glycoprotein BEH™ Amide (2.1 mm × 50 mm, 1.7 μm, 300 Å) column. For IEX analysis, BioResolve SCX mAb Columns (commercial 2.1 mm × 50 mm and prototype 2.1 mm × 15 mm, 3 μm) were used. SEC analysis was performed using the ACQUITY Premier Protein SEC (4.6 mm × 150 mm, 1.7 μm, 250 Å) Column.

Data processing and retention modeling was performed using DryLab™ 4.4 Software (Molnar Institute, Berlin, Germany). A 2² full factorial design applied for SEC optimization was evaluated with Statistica™ 14 (TIBCO) software.

2.3. Mobile phase compositions and sample preparation

For RPLC separations, 0.1 % DFA in water and 0.1 % DFA in ACN were used as mobile phase A and B, respectively. HILIC separations were performed with a stronger additive in the mobile phase, namely 0.1 % TFA in ACN for mobile phase A and 0.1 % TFA in water for mobile phase B. The salt-mediated IEX gradients were performed with 10 mM MES pH 6.0 (mobile phase A) and 10 mM MES with 400 mM potassium chloride pH 6.0 (mobile phase B). For the pH-gradient IEX separations, ten-fold diluted BioResolve CX pH Concentrate A (pH 5) and BioResolve CX pH Concentrate B (pH 10.2) were used as mobile phase A and B, respectively. SEC analysis was performed using a phosphate buffered saline (PBS) pH 7.4 buffer [25] and methanol as organic modifier in mobile phase B.

Protein subunits were generated by adding 120 units of *IdeS* enzyme to 100 μg of mAb in a solution of 10 mM Tris buffer (pH 7.3) [26]. The final concentration of 1 mg/mL was incubated for 30 min at 45 °C. Subsequently, for the reduction of protein material, 100 mM of DTT was added to the digest, and it was incubated for 30 min at 45 °C. The sample material was then directly analyzed. Only for HILIC analysis was a further dilution applied to avoid peak distortion upon injection (4 parts digest to 1 part 0.1 % TFA acidified acetonitrile). Intact analysis was performed by dilution with water to 1 mg/mL prior to injection. An injection volume of 1 μL (1 mg/mL sample material) was used unless stated otherwise.

2.4. Systematic method optimizations

Method optimization was performed on the basis of critical resolution maps generated by DryLab Retention Modeling Software. This allowed for the rapid identification of optimal method conditions based on 2D retention models. The 2D models were created from only 4 initial experiments in which two method variables, such as, gradient steepness (gradient time, t_G), mobile phase temperature (T) or amount of organic modifier in the mobile phase (%B), were considered and included to build up the design space.

For RPLC analysis, a preliminary generic linear gradient experiment was run from 25 % to 45 %B in 10 min at a flow rate of 0.6 mL/min and 70 °C to see whether all compounds could be eluted in a reasonable retention window ($1 < k_{app} < 15$). Then, based on the preliminary runs, the method variables to calibrate the 2D RPLC model were selected as

follows: $t_{G1} = 4$ and $t_{G2} = 12$ min; $T_1 = 65$ and $T_2 = 90$ °C.

For the IEX separations, pH-mediated and salt-mediated gradients were directly compared. For both gradient methods, first a generic scouting linear gradient was run from 0 % to 100 %B in 20 min at 0.3 mL/min flow rate and at $T = 25$ °C. Based on these results, a 2D retention model was considered with the combination of t_G and T. The method variables selected were: $t_{G1} = 8$ min and $t_{G2} = 20$ min; $T_1 = 25$ and $T_2 = 45$ °C (please note that for IEX separations non-denaturing conditions are considered to be maintained, thus only moderate temperature can be applied). The gradients were run as 0 – 50 %B, since all species eluted in this gradient range.

For HILIC analysis, a similar 2D retention model was considered and created by applying a gradient of 15–35 %B gradient (with MPB being 0.1 % TFA in water) and setting gradient time to $t_{G1} = 4$ and 1 $t_{G2} = 12$ min, and column temperature to $T_1 = 65$ and $T_2 = 90$ °C. Flow rate was set as 0.6 mL/min and 0.5 μL of 0.8 mg/mL protein material (dissolved in 20 % of MPA) was injected. After the optimal conditions were found, an initial short peak compression step was added - prior to the analytical gradient - in which the %B was rapidly increased (in 0.5 min) from 20 % to starting conditions of the gradient (to avoid break-through effects and peak distortion [27]).

For the SEC analysis, a preliminary scouting experiment was performed at a flow rate of 0.3 mL/min (run time set as 8 min) and with 2 % of methanol in the mobile phase and at $T = 25$ °C. To reduce possible non-desired hydrophobic – secondary - interactions, 2–15 % (v/v) concentrations of organic solvent is often added to the mobile phase [28, 29]. To find the appropriate organic solvent strength for accurate quantitation of aggregates and fragments, we considered the methanol concentration of the mobile phase (%B) as a method variable. On the other hand, temperature was considered as the second variable as it can also impact non-desired secondary interactions and thus aggregate recovery and peak shape. Based on the obtained preliminary chromatographic profile, the following method variables were selected for the method optimization: $T_1 = 25$ and $T_2 = 40$ °C; amount of organic modifier $\%B_1 = 2$ % and $\%B_2 = 10$ %.

RPLC, IEX and HILIC optimization was performed on commercial 2.1 × 50 mm columns, while the SEC optimization was performed on a 4.6 × 150 mm column. After optimizing the RPLC and IEX separations, the methods were transferred to ultra-short columns (2.1 × 20 mm for RPLC and 2.1 × 15 mm for IEX).

2.5. Automation of the optimization procedure and chromatographic experiments

A recently introduced automation module within DryLab Software was utilized to fully automate the method optimization process for all chromatographic modes. This new module supports the direct communication and full control between the modeling software and the instrument controlling chromatographic data system (CDS, in this case Empower Software). Once a retention model is selected - to be calibrated (e.g. $t_G - T$ model) -, all the instrument method parameters, method sets and sample set parameters can quickly be set within the modeling

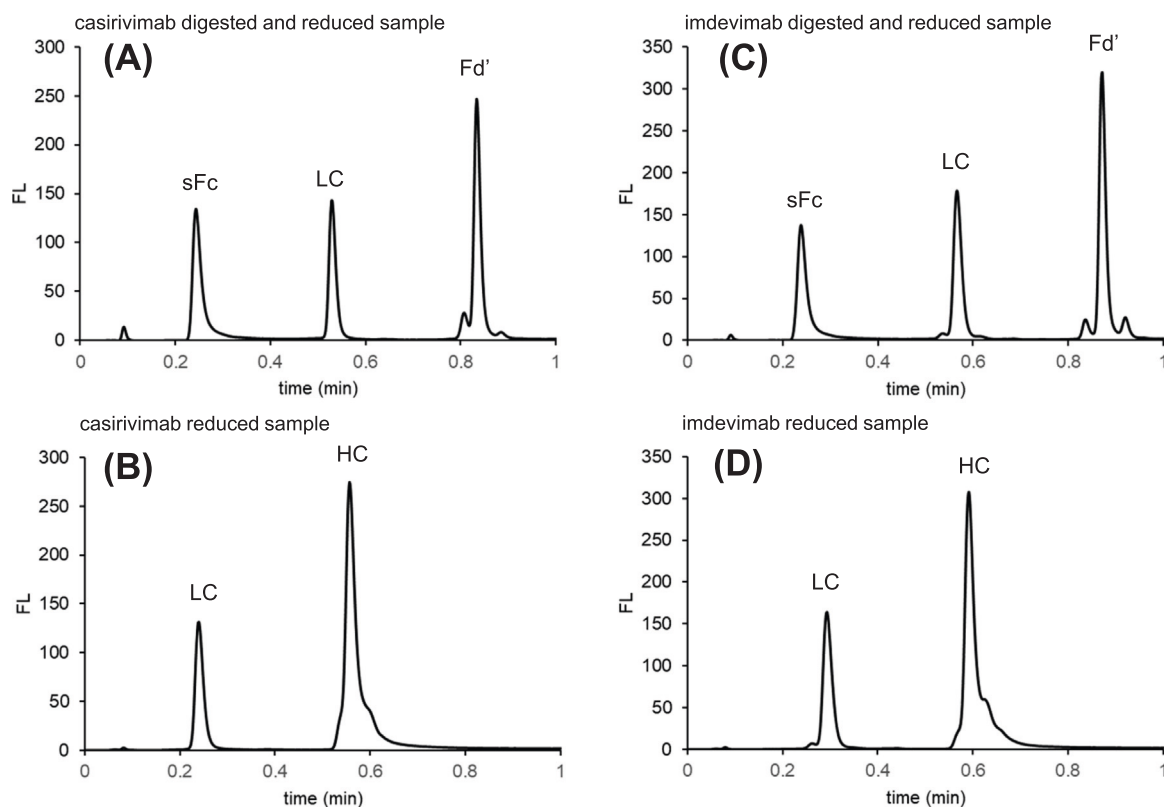


Fig. 2. Optimized separation conditions for RPLC analysis of casirivimab (A,B) and imdevimab (C,D) subunits. Column: 2.1×20 mm, flow rate $F = 1.3$ mL/min; gradient: 26–36 %B in 1 min (for digested and reduced samples, A and C), 29–37 %B in 1 min (for reduced only samples, B and D); $T = 75$ °C; injected volume: 0.3 μ L; MPA: 0.1 % DFA in H_2O ; MPB: 0.1 % DFA in AcN.

software (including column equilibration times, washing steps, repetitions and blank injections as well). Then, the whole experimental design and corresponding instrument parameters created in the modeling software can be transferred to the CDS. Once the experiments are performed, all measured data required to calibrate the retention models (e. g. retention times, peak areas, peak widths, symmetry) can be directly sent from the CDS to the modeling software and the models can be quickly set up. After selecting a robust working point (in-silico optimization, without the need to perform further experiments), the corresponding conditions and method parameters can be transferred again to the CDS in order to automatically perform the experimental verification of the selected working point. Fig. 1 shows a schematic view of the automation procedure.

2.6. Transfer of the optimized methods to ultrashort columns

The optimized RPLC and IEX methods (developed on 2.1×50 mm columns) were virtually transferred to 2.1×20 mm (RPLC) and 2.1×15 mm (IEX) columns considering intended changes in system gradient delay volumes and applied flow rates and as well as taking the column length changes into account. DryLab Software was applied to perform the virtual method transfers [30]. Optimization runs were performed with a quaternary system (to benefit from more potential mobile phase composition) while the ultra-short columns (transferred methods) were used with an optimized binary system (optimized for extra-column dispersion). On ultra-short columns, very small column peak variances and short analysis times (e.g. ≤ 2 min) are expected, therefore extra-column band broadening and gradient delay of the system should be minimized to obtain the full benefit of ultra-short columns [24]. First, the optimal conditions found on 2.1×50 mm columns were geometrically transferred to 2.1×20 mm (RPLC) and 2.1×15 mm (IEX) columns. The virtual geometrical transfer then was experimentally

verified. We noticed that the observed separation quality was at least equivalent - or even better - on the ultra-short columns (using the low dispersion system), therefore attempts were made to further shorten the analysis time. Hence, flow rate and gradient time were changed (maintaining identical or very similar intrinsic gradient steepness to not affect selectivity) to target the 1–2 min analysis time interval. Some conditions were tested in-silico and then the most promising conditions experimentally verified. Finally, the predicted (calculated, virtually transferred) retention times were compared to the experimentally measured retention times to study the reliability of the virtual method transfer.

3. Results and discussion

3.1. RPLC analysis of casirivimab and imdevimab subunits

There has been a pronounced trend in RPLC analysis of mAbs that favors a so-called middle-up approach, which consists of a separation of reduced light chain (LC) and heavy chain (HC) variants or the analysis of Fc, sFc, Fab, $F(ab')_2$ or Fd' fragments that can be created by limited proteolysis [31]. Various chemical and post-translational modifications can be quickly detected and monitored by RPLC via a subunit level analysis. Separations for this are often performed at elevated temperature ($T > 60$ °C) to obtain sharpened peak shapes and improved recoveries.

The effect of mobile phase temperature and gradient steepness were studied based on 2D critical resolution maps. In case of all four samples (reduced casirivimab, reduced imdevimab, digested and reduced casirivimab, and digested and reduced imdevimab), mobile phase temperature had only a slight impact on resolution while gradient steepness was found to be a critical variable (as predicted). Regarding temperature, we tried to maintain this parameter as low as possible to avoid artificial

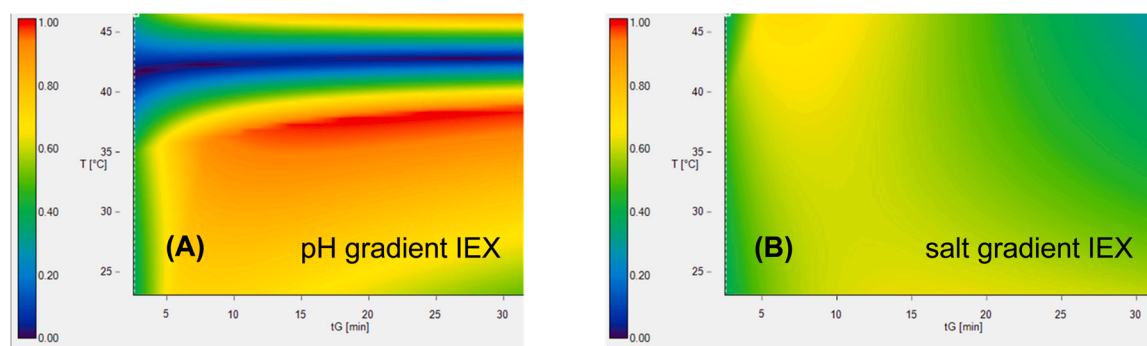


Fig. 3. Dual design space comparison (critical resolution as function of t_G and T) for pH- (A) and salt-gradient (B) CEX analysis of partially digested imdevimab.

sample degradation. However, at 65 °C, slightly lower peak areas and broadened peaks were observed compared to 90 °C. We found that 75 °C was a good compromise as it resulted in complete peak recovery and relatively narrow peak widths. For mobile phase gradient optimization, we attempted to set an equidistant peak distribution within the elution window of the method. It was found that linear gradients of 26–36 %B (for the digested and reduced samples) and 29–37 %B (for the reduced samples) provided nearly uniform selectivity for the main species and acceptable resolution of some minor variants. (The chromatograms observed with the 2.1 × 50 mm commercial column are shown in [Supplementary Information's Fig. 1.](#)) Then, using the separation models, these gradients were virtually transferred to a 2.1 × 20 mm column and to a system with $V_d = 0.110$ mL. Experimental verification showed very good agreement between the modeled (virtually transferred) and measured (experimentally transferred) chromatograms. The average error of retention time prediction was lower than 1.5 %. As a final step of

optimization, gradient time was further decreased along with a proportional increase of the flow rate to quicken the analysis time. In the end, it was found that a $t_G = 1$ min gradient at $F = 1.3$ mL/min flow rate provided sufficient separation for all four samples. [Fig. 2](#) shows the final optimized chromatograms obtained on an ultra-short (2.1 × 20 mm) RPLC column. ([Supplementary Information's Figs. 2 and 3](#) show the virtually transferred and experimentally measured chromatograms for the 2.1 × 20 mm column and the relative errors of retention time prediction.)

3.2. IEX analysis of casirivimab and imdevimab at intact and at subunit levels

Cation-exchange chromatography (CEX) is usually employed for charge variant analysis and to identify and quantify acidic versus basic species [32]. In CEX, two approaches are often used to elute antibody

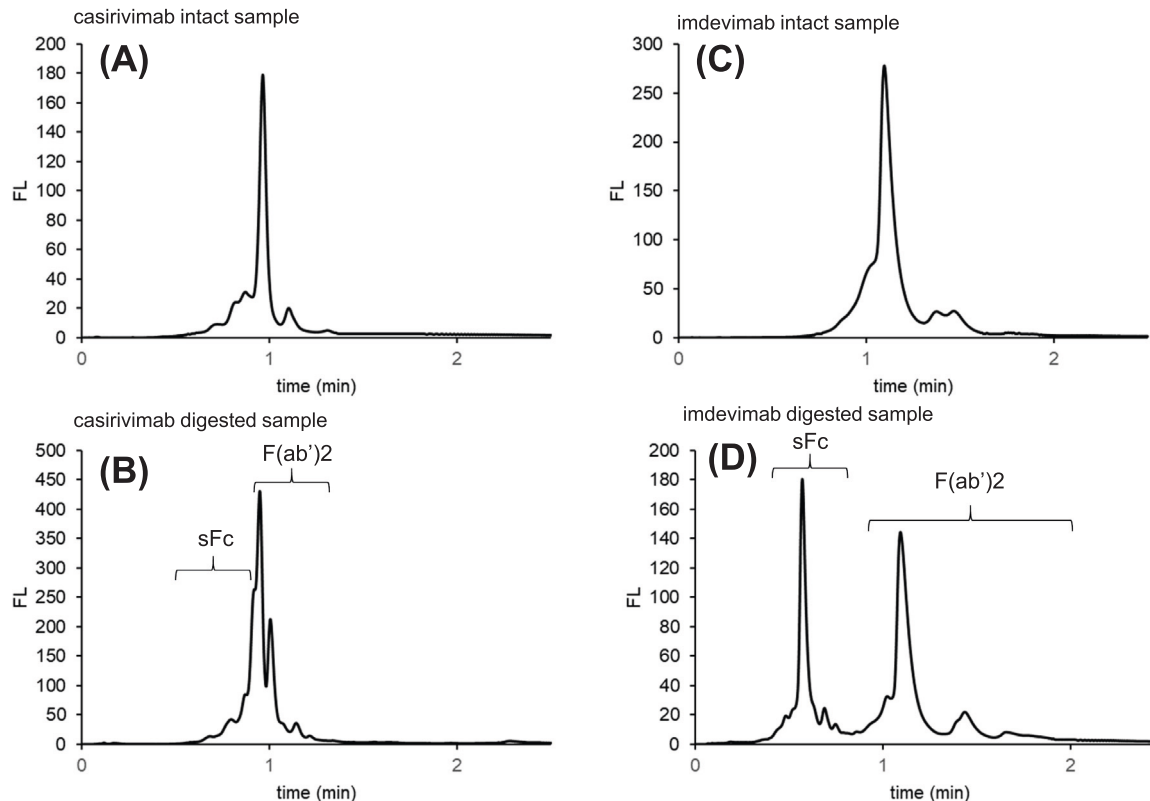


Fig. 4. Optimized separation conditions for CEX analysis of intact (A,C) and digested (B,D) casirivimab (A,B) and imdevimab (C,D). Column: 2.1 × 15 mm, flow rate $F = 0.5$ mL/min; gradient, 10–30 %B in 2 min for intact casirivimab (A), 0–25 %B in 2 min for digested casirivimab (B), 15–35 %B in 2 min for intact imdevimab (C), 5–35 %B in 2 min for digested imdevimab (D); $T = 25$ °C; injected volume: 0.5 μ L, MP: ten-time diluted BioResolve CX pH Concentrate A (pH 5) and B (pH 10.2). Peak identification requires further experiments.

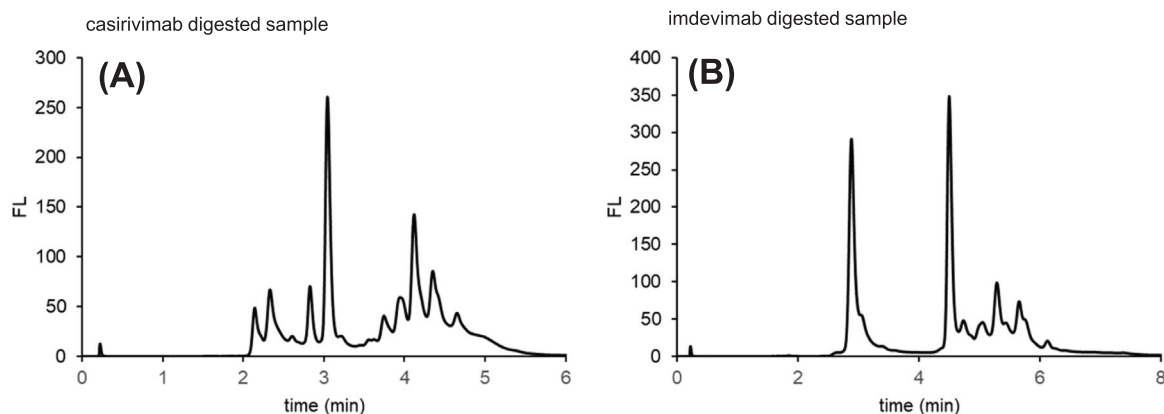


Fig. 5. Optimized separation conditions for HILIC analysis of casirivimab (A) and imdevimab (B) subunits. Column: 2.1×50 mm, flow rate: $F = 0.7$ mL/min; gradient: starts as 20 %B – 26 %B in 0.5 min then 26–34 %B in 5 min (5.5 min in total); $T = 65$ °C; injected volume: 0.5 μ L; MPA: 0.1 % TFA in AcN; MPB: 0.1 % TFA in H_2O . Peak identification requires further experiments.

species. One consists of weakening the strength of ionic interactions between the analyte and the stationary phase by increasing the ionic strength of the mobile phase. This mode is referred as a salt-gradient technique. The other mode is based on changing the pH of the eluent in time, in order to decrease the net charge of the solute to thus promote their elution. This latter approach is often termed as a pH-gradient separation. The two different gradient modes might result in different selectivity or elution profiles; therefore, we were interested in comparing them. The 2D resolution maps were systematically compared for four samples consisting of intact casirivimab, intact imdevimab, partially digested casirivimab and partially digested imdevimab. For a direct comparison of the two modes, we applied a module within DryLab Software for “design space comparisons”. This feature of the modeling software is originally designed to compare the resolution maps of complete separation systems, for example, separations obtained on different columns using a single type of separation mode [33]. However, here, our idea was to exploit a design space comparison in a different way; namely in a way that would compare one column operated in two different elution modes. For the most part, the two modes provided similar elution profiles. Yet, slightly better separations of basic species were achieved by pH gradient elution especially for the digested imdevimab sample (see in [Supplementary Information’s Fig. 4](#)). Therefore, the pH gradient mode was selected and individually optimized for each sample. [Fig. 3](#) shows a representative plot of design space comparisons between pH- and salt gradient modes for partially digested imdevimab. As suggested by the resolution maps, an overall higher critical resolution could be obtained with the pH gradient ($Rs_{crit} = 0.98$) versus the salt gradient technique ($Rs_{crit} = 0.69$). IEX separations are complicated and baseline resolution is hard to achieve for many critical charge variants. In fact, shoulder peaks for acidic and basic variants are frequently encountered, but an Rs value of 1 is closer to what is desired and cited by the United States Pharmacopeia in their guidelines on LC method system suitability (e.g. an Rs value of at least 1.5).

After finding the optimum conditions on commercial 2.1×50 mm columns, the methods were virtually transferred to 2.1×15 mm ultra-short columns and to a low dispersion chromatographic system with a low gradient delay volume. Similarly to the RPLC method transfer, gradient time was further decreased while applying a proportional increase of the flow rate. Finally, it was found that a $t_G = 2$ min gradient at $F = 0.5$ mL/min flow rate worked well for all four samples. The following gradient programs were set to optimize these CEX profiling methods: 10–30 %B for intact casirivimab, 0–25 %B for partially digested casirivimab, 15–35 %B for intact imdevimab and 5–35 %B for the partially digested imdevimab. Temperature was set at $T = 25$ °C since elevated temperature did not bring any substantial improvement in resolution. [Fig. 4](#) shows the optimized chromatograms obtained on a

ultra-short (2.1×15 mm) CEX column. The average error of retention time prediction for the in-silico method transfer was lower than 3 %. ([Supplementary Information’s Figs. 5 and 6](#) show the virtually transferred and experimentally measured chromatograms for the 2.1×15 mm column and the relative errors of retention time prediction.).

3.3. HILIC analysis of casirivimab and imdevimab subunits

HILIC is a very efficient tool for the analysis of mAbs glycan heterogeneity, especially when put to practice at the subunit level. Compared to RPLC, HILIC can often separate additional peaks, and the sFc fragments normally elute in multiple peaks due to the separation of their glycovariants [34]. In many cases, the elution order of the fragment species is reversed compared that of RPLC. This is not strictly predictable, though, given the complex retention mechanisms in HILIC. MS based identification of peaks from HILIC subunit separations thus plays a key role in confirming peak identifications [34].

After performing the calibration runs, HILIC separations were also optimized by means of their critical resolution maps. Peak areas did not change significantly with temperature, suggesting appropriate recovery was already achieved at $T = 65$ °C. Moreover, selectivity and resolution were comparable across the entire temperature range. The optimized method employed $T = 65$ °C, as a result. When optimizing gradient steepness and initial- and final mobile phase compositions, it was found that a linear gradient of 26 – 34 %B in 5 min at $F = 0.7$ mL/min resulted in appropriate separation for both samples. Finally, an initial short steep gradient ramp was added - prior to the analytical gradient - to avoid potential breakthrough effects and peak distortion that can occur with larger injection volumes and more concentrated samples. [Fig. 5](#) shows representative HILIC chromatograms obtained on 2.1×50 mm HILIC columns.

The use of prototype ultra-short HILIC column hardware designs were tested here on this separation too. However, the apparent plate heights of these columns were slightly lower compared to those obtained on longer, commercial columns. Additional work is needed to better define column manufacturing practices and the practical limits of applying short bed lengths to protein HILIC separations. Ultimately, the presented here achieves a ~5–6 min long HILIC analysis of antibody subunits on a 2.1×50 mm column. In itself, this is a sizeable improvement in method throughput. To date, mAb subunits have generally been analyzed on 2.1×150 mm HILIC columns using methods with 10–20 min run times [34].

3.4. SEC analysis of casirivimab and imdevimab

SEC is an important reference method for the analysis of protein size

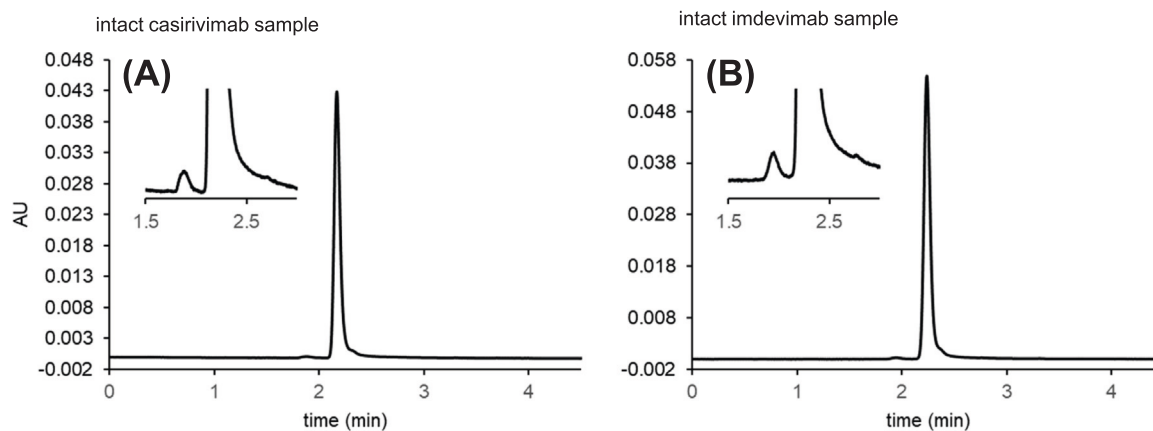


Fig. 6. Optimized separation conditions for SEC analysis of intact Casirivimab (A) and Imdevimab (B). Column: 4.6×150 mm, flow rate $F = 0.5$ mL/min; analysis time: 4.5 min; $T = 25$ °C; injected volume: 1 μ L; UV detection at 280 nm. MP: 1 \times PBS buffer, pH = 7.4.

variants, including both aggregates/high molecular weight species (HMWs) and low molecular weight fragments. An ideal size exclusion column would exhibit no adsorptive interactions between its internal surfaces (frit and column wall) and analytes. Recently, a new packing material (hydroxy terminated polyethylene oxide) has been developed and packed into column hardware modified with hydrophilic hybrid surface technology (h-HST) [35]. This new column showed clear benefit to obtaining higher apparent recoveries to better ensure accurate aggregate quantitation and also resulted in more symmetrical peak shapes compared to other state-of-the-art packing materials and column hardware. Therefore, this column (ACQUITY Premier Protein SEC Column 250 \AA $1.7 \mu\text{m}$) has been selected for this study. A more traditional 4.6×150 mm column configuration was used for all experiments. That there is no adsorption in SEC means that separation power will always be directly correlated with increases in column length. Nevertheless, a 150 mm long column is on the shorter side of column lengths selected for profiling experiments, where a 300 mm dimension is more frequently relied upon. A sub- $2 \mu\text{m}$ packed bed also provides amenability to faster flow rates.

The automation module of the DryLab Software was applied to automate the experiments. Simultaneous effects of method factors (T, % B) were evaluated by means of a simple full factorial (2^2) design. Three response functions have been selected, such as: (1) recovery of HMWs, (2) elution time of the monomer peaks and (3) width of the monomer peaks. These responses can be indicators of non-desired (non-specific) secondary interactions. It was found that neither temperature (T) nor the concentration of organic modifier in the mobile phase (%B) had significant effect on any responses. These results suggest that secondary interactions are practically negligible in the studied range of method factors. At the same time, the resolution between HMWs and monomer peaks were found to be greater than 1.5, indicating that the pore size of the packing material was also properly selected (250 \AA).

For the final method, a flow rate of $F = 0.5$ mL/min and $T = 25$ °C were set and pure aqueous mobile phase (1 \times PBS buffer, pH = 7.4) was selected. Please note, that initial experiments were performed at $F = 0.3$ mL/min, but flow rate was then increased in order to decrease analysis time without causing any deterioration in separation quality. $F = 0.5$ mL/min still provided baseline resolution, sharp peaks and an analysis time shorter than 4.5 min Fig. 6 shows representative SEC chromatograms for the two mAbs. For imdevimab, we observed 0.63 % HMWs while for casirivimab 0.73 % HMWs was measured.

4. Conclusion

This work presents the benefits of combining retention modeling, automated experiments, and the use of short protein columns for mAb analysis.

Retention modeling enables the *in silico* optimization of mAb (or any protein) separations within a few minutes. The automation module of DryLab Software significantly simplifies manual work and thus speeds up the method development procedure. The whole process to develop and optimize four chromatographic methods (RPLC, IEX, HILIC and SEC) for two mAb samples might require only 2 days of experimental work.

By using short protein columns (15–20 mm long for RPLC and IEX), analysis times in the range of 1–2 min were obtained, marking a significant time savings in comparison to 10–30 min long traditional methods. Overall, we have shown that conventional chromatographic techniques can quickly be transferred *in silico* to short column, higher throughput parameters and to low dispersion chromatographic systems without the need for further experiments. These approaches might be valuable for turning around scientific data on drug candidates during critical times when the preparation of filing information must be accelerated.

CRediT authorship contribution statement

Bastiaan Duivelshof: Investigation, Writing – original draft. **Arnold Zöldhegyi:** Conceptualization, Writing – original draft. **Davy Guillaume:** Writing – review & editing. **Matthew Lauber:** Writing – review & editing. **Szabolcs Fekete:** Methodology, Conceptualization, Writing – original draft.

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. Columns and LC instruments used in this research were paid for and made available by Waters Corporation.

BioResolve, ACQUITY, UPLC, Empower and BEH are trademarks of Waters Technologies Corporation. FABRICATOR is a trademark of Genovis AB. Milli-Q is a trademark of Merck KGAA. DryLab is a trademark of Molnar Institute. Statistica is a trademark of TIBCO Software Inc.

Data Availability

The data that has been used is confidential.

Acknowledgement

The authors wish to thank Jean-Luc Veuthey from the University of Geneva for his fruitful comments and discussions during the preparation of this manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jpba.2022.115039](https://doi.org/10.1016/j.jpba.2022.115039).

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