Development of an automated method for monoclonal antibodies purification and analysis

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Two major problems in biotherapeutics are aggregation of and the presence of variants of active pharmaceutical ingredients. These product-related substances can have different efficacy than the main product and may cause serious side effects, for example, anti-drug-antibody formation. Protein aggregates are mostly the consequence of suboptimal production, purification or handling conditions.

This article discusses the development of an automated solution for purification and separation (by ion exchange or size-exclusion chromatography) of antibodies in a single method. In this process the autosampler of the HPLC was configured to performs the injection, high volume fraction collection, and reinjection.

Keywords: antibody purification, multidimensional workflows, 2DLC, automation, monoclonal antibodies

1. Introduction

During various stages in the development of biopharmaceuticals, purification, and analytical characterisation of the product are required. In the early phase of process optimisation of recombinant antibodies, a large number of samples must be screened for titer, aggregation, and protein variants. These protein aggregates and variants of the active pharmaceutical ingredient can have different efficacies than the main product and may cause serious side effects, for example, anti-drug-antibody formation.

Protein aggregates are mostly the consequence of suboptimal production, purification, or handling conditions (temperature or pH). In the purification of antibodies, a protein affinity separation is generally the first step. Affinity chromatography on protein A or G columns typically yields a purity of more than 95% in a single step. While the purification on affinity columns yields information on the titer of the product, it is unselective with respect to related substances.

To verify the sample purity or antibody quality, techniques, such as ion exchange chromatography (IEX) or size-exclusion chromatography (SEC) are needed. SEC provides the necessary selectivity to identify agglomerates and size-based variations of the main component. Ion exchange stationary phases provide good selectivity for separation of charge variants of the protein biopharmaceutical. The variations may be very subtle or small and finding the optimal chromatographic conditions requires optimization.

This article discusses an automated solution for purification followed by a separation (IEX or SEC) of antibodies using the UltiMate® 3000 Titanium HPLC system (Figure.1). In this process, multiple protein separation steps are performed automatically. The autosampler performs the injection, high-volume fraction collection, and reinjection of the collected fractions for analysis.

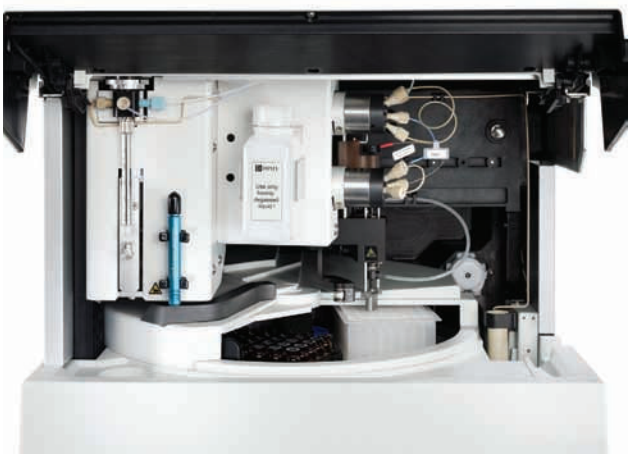

Figure 1. (A) The UltiMate 3000 Titanium solution with (B) the WPS-3000TBFC bio-inert autosampler/fractionation module.

2. Instrumentation and 2DLC workflow

HPLC experiments were carried out using an UltiMate 3000 Titanium (Dionex Corporation) equipped with:

- SRD-3600 Membrane degasser
- DGP-3600MB x2 dual-gradient pump system (Alternative: DGP-3600AB, Analytical pump)
- TCC-3000SD Thermostatted column compartment with valve actuators and biocompatible pods
- WPS-3000TBFC Analytical dual valve well-plate sampler (Fig.1B)
- VWD-3400RS UV detector equipped with an 11 µL flow cell

The Dionex Chromeleon® extended fractionation software driver was added in the server configuration. This is required for controlling the advanced features of the WPS-3000TBFC. This software extension is capable of managing time- and peak-based fractionation including a wide range of settable parameters to adapt the fractionation to the specific needs of the analysis. Fractionation can be operated in continuous mode or pooled mode as used in enrichment experiments.

The WPS-3000TBFC

This dual-valve well plate autosampler is capable of fractionating into various types of vials and well-plates. It can operate at elevated flow rates by using the second valve as a diverter valve. The dual needle design with a metal pre-puncture needle allows fractionation in both capped as uncapped vials and well plates, as well as eppendorf tubes. Both puncture depth and sample needle protrusion depth can be programmed for each sample container format. In addition to this, the WPS-3000TBFC also offers sample handling and derivatization capabilities (for example, in well digestion, pH adjustment, dilution). It has a thermostatted sample compartment (4-45°C) and easy-to-adapt fluidics to suit application needs, including full-loop injection volume ranges from 1 to 500 µL. Also, it accommodates multiple well plate types simultaneously. Finally, the modules' excellent repeatability for injection and fractionation provides a robust platform for any analysis.

The workflow and LC conditions for automated off-line 2DLC included the following:

- Injection of 50 to 250 µL of an unpurified monoclonal/polyclonal antibody sample.
- A first-dimension (1D) Affinity Chromatography separation, at a flow rate of 1.5 mL/min using the following steps:
  - A column wash/equilibration step of 2 min
  - An elution step of 5 min
  - Automated peak detection followed by fraction collection into a well plate in the autosampler
- A second dimension (2D) separation (one of the following):
  - Weak cation exchange separation at pH 5.5, applying a linear NaCl salt gradient
  - Size-exclusion chromatography separation (200 mM Sodium Phosphate, 250mM NaCl, pH 6.3)

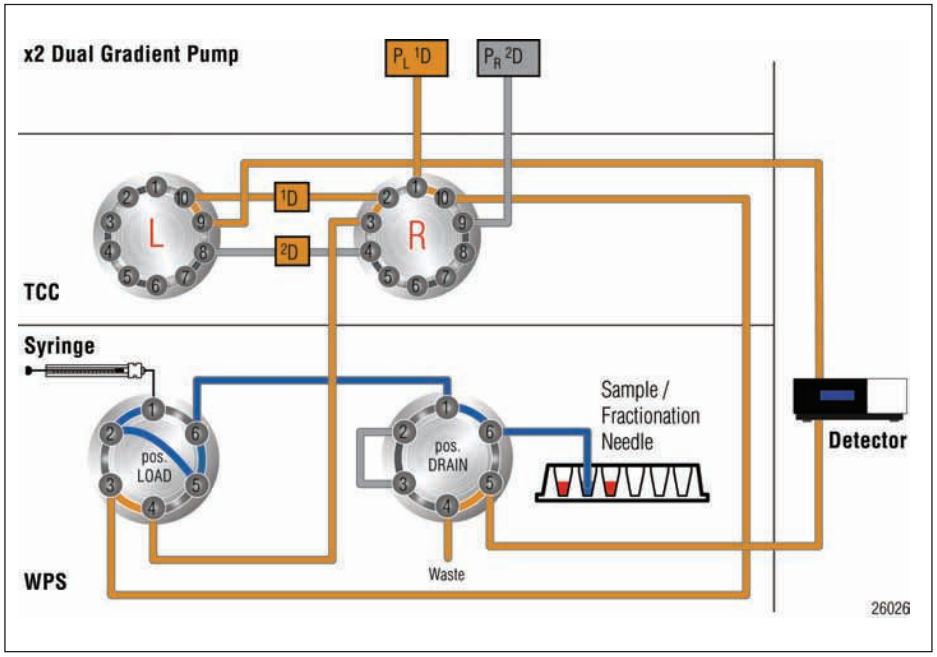

Figure 2. Fluidic setup of the automated offline 2DLC system using the WPS-3000TBFC

(Prior to the 2D separation, an optional neutralization buffer can be added using the derivatization capabilities of the autosampler to raise the pH of the fraction.)

3. LC conditions

Protein A affinity separation

A protein A column (AB, Poros 20 µm, 4.6 mm i.d. x 50 mm, 0.8 mL) was used for first dimension affinity based separations with UV detection at 214 and 280 nm. Between 50 and 250 µL of sample was applied, making use of the large volume injection kit and a User Defined Program (UDP) to fill the sample loop. Affinity LC conditions: mobile phase A: 10 mM Sodium Phosphate, 150 mM NaCl, pH 7; mobile phase B: 50mM Glycine-HCl, 150 mM NaCl, pH 2.5. 1D dimension wash and equilibration step for 2 min at 100%A, followed by a 5 min wash step at 100%B. Flow rate: 1.5 mL/min, column temperature: 25°C.

Ion-exchange separation of protein variants

A ProPac® WCX-10 column (2 mm i.d. x 250 mm, Dionex) was used for the 2D weak-cation-exchange LC separation applying a linear salt gradient of MES buffer/sodium-chloride solution, detection at 214 and 280 nm. Mobile phase A: 20 mM MES buffer pH 5.5 + 60 mM NaCl; mobile phase B: 20 mM MES buffer, pH 5.5 + 180 mM NaCl. The 2D dimension gradient on this column went from 45 to 85 %B (50 min), 6 min wash step at 100%B, and 12 min equilibration time at 45%B. Flow rate: 250 µL/min, column temperature: 25°C.

Size exclusion separation of protein aggregates

A SEC column was used for the Aggregation analysis (4.6 mm i.d. x 300 mm, BioLC, Japan), the mobile phase used was 200 mM Sodium Phosphate, 250 mM NaCl, pH 6.3.

4. Results

Typically, the antibody is manually injected onto the affinity column. Here, the autosampler was used to introduce the sample to this column. After an equilibration step with mobile phase A, the antibody was eluted from the Protein A column with mobile phase B and fractionated into a 96-deep-well plate using peak based triggers (Figure 3). These triggers can be easily optimized to either collect or discard the breakthrough peak and to collect the antibody peak with a trigger based on retention time and signal properties. There is also some room for speeding up this process as the pump module can deliver up to 6 mL/min. However, a minimum fractionation/decision delay time (5 s) should be considered. If required, a neutralizing buffer can be added to the fractions after

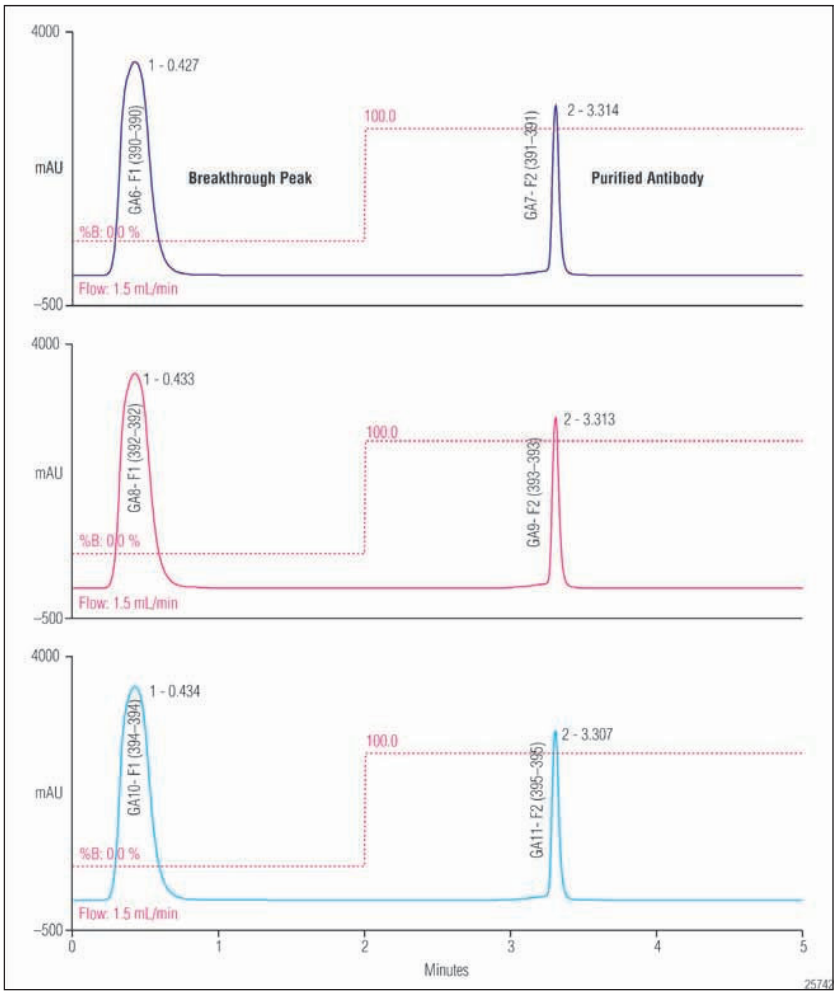

Figure 3. First-dimension affinity chromatography separation of a polyclonal antibody; 0-2 min: wash step; 2-5 min: elution step. This set of chromatograms also illustrates the repeatability of the injection and automatic peak detection functionalities.

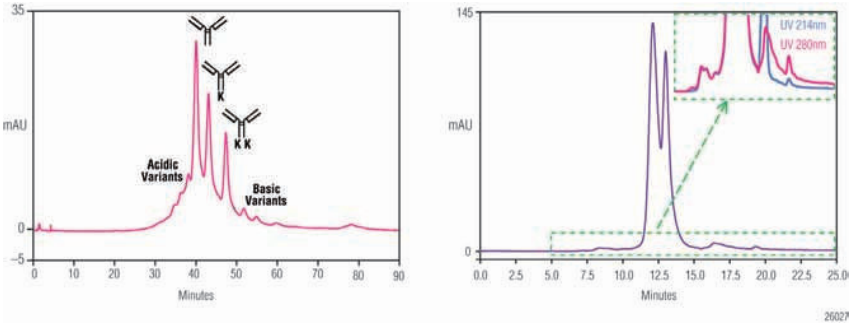

Figure 4. (A, left) Example of a second dimension IEX separation (ProPac WCX-10, 250x4.6 mm I.D.) of a purified monoclonal antibody fraction. (B, right) Example of a second dimension SEC separation of a purified polyclonal antibody fraction.

fractionation to increase the pH, using the derivatization capabilities of the autosampler (Figure 4). For the antibody peak, a peak area RSD of 0.037% and a retention time RSD of 0.115% (tR= 3.31) was found.

When a fraction is found that meets the requirements, the Chromeleon software can be used to initiate a post-acquisition step for the second dimension separation. This can be a weak cation exchange based separation, yielding selectivity towards small charge differences in the antibody sample (Figure 4A, IEX of a monoclonal IgG1 fraction), or an SEC to separate the antibody from its di-mers, aggregates, and so on (Figure 4B, SEC of a polyclonal antibody). In the IEX chromatogram, we clearly see the heavy chain C-terminal lysine heterogeneity variants of the mAb, described by the three largest peaks. Also a series of basic and acidic variants are visible. A more detailed look into monitoring monoclonal antibody heterogeneity and stability by IEX can be found in AN127 and AN128 (1,2).

In the applied workflow, all first-dimension separations are carried out, followed by the automatically generated second-dimension separation sequence. The sample throughput can be further increased by speeding up the flow rate for the affinity and the SEC separation and shortening the different steps required in the affinity separation. Also, the IEX separation may be accelerated by modifying the gradient.

5. Conclusions

The dual-valve WPS-3000 TBFC autosampler enables high flow fraction collection and reinjection of fractions as used in 2D workflows. Such workflows include automated offline two-dimensional column coupling (for example, Affinity with IEX or SEC; RP with RP utilizing different pH levels; SPE with RPLC); as well as IEX with fractionation, trapping and desalting prior to MS detection. It is clear that this module can serve as a platform for coupling various chromatographic techniques for biopharmaceuticals and proteins analysis.

The Chromeleon software package allows this degree of automation with a wide range of flexibility to accommodate the most demanding applications. Sample handling and derivatization, purification, and HPLC analysis can be programmed both easily and in depth and gives the instrument unique capabilities in the field of sample purification, standard HPLC and QC.

6. References

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