A Good Fit?

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UHPLC systems have already made their way to routine labs for the analysis of small molecules and APIs, but are established bioanalysis methods and proven BioHPLC columns also compatible?

Keywords

Ultra-high-pressure liquid chromatography (UHPLC)
High-performance liquid chromatography (HPLC)
Protein aggregation
Gel filtration
Bioseparation method transfer

Besides tolerating application pressure exceeding 1,000 bars, an ultra-high-pressure liquid chromatography (UHPLC) system is, first of all, high-performance liquid chromatography (HPLC) optimised with regards to dead volume, injection performance and detector sampling rate. From that perspective, it should not only be possible, but even advantageous, to use UHPLC instrumentation for existing bioseparation methods, initially developed with HPLC columns on conventional HPLC systems. Some examples of typical HPLC applications frequently used in characterisation and quality control of biologics illustrate the potential of using UHPLC instruments in bioseparation.

Analysis of Protein Charge Variants

Apart from isoelectric focusing, cation exchange chromatography is the method of choice to analyse the

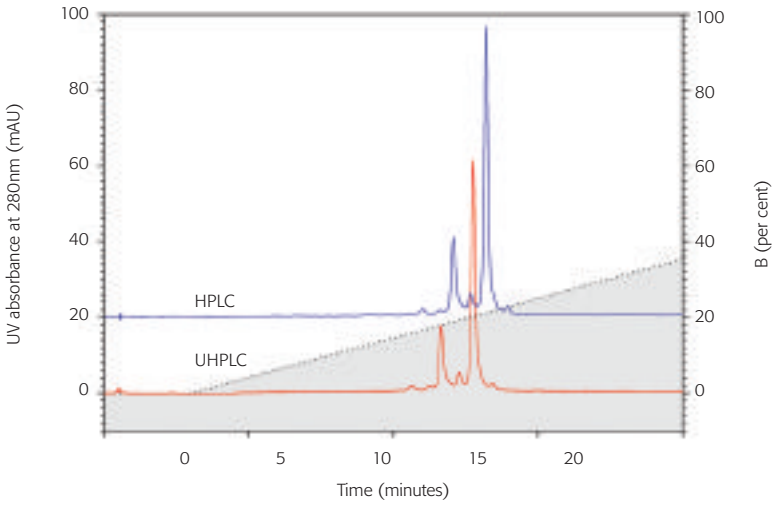

Figure 1a: A monoclonal IgG was analysed using TSKgel SP-STAT 4.6mm ID x 10cm L. For analysis, a HPLC system (blue) and a UHPLC system (red) of the same manufacturer were used.

Flow: 1mL/min; Mobile Phase A: 10mM sodium phosphate, pH 7.0; B: 10mM sodium phosphate, pH 7.0 + 1M sodium chloride; Gradient 0-50 per cent B in 25 minutes; Detection UV at 280nm; Injection volume 5µL each; Injected mass 5µg

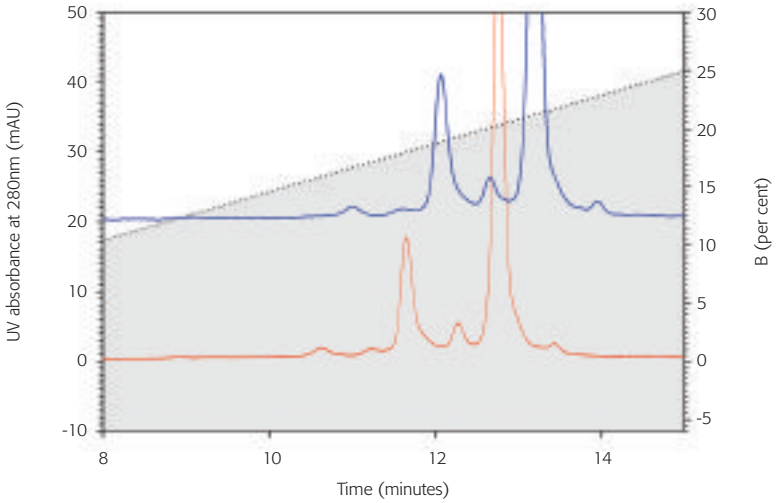

Figure 1b: Zoomed elution profile of the mAb charge variants on TSKgel SP-STAT. The zoom provides better insights into what extent the elution profile benefits from using a UHPLC system. The peak width is smaller for the UHPLC elution profile, compared to the HPLC chromatogram. Due to the decreased system dead volume, peak elution occurs earlier than for the HPLC system

Source: Tosoh Bioscience

charge heterogeneity of proteins. Charge isoforms of proteins result from deamidation of asparagine or glutamine residues, or from incomplete removal of C-terminal lysine residues. A monoclonal antibody (mAb) and its charge variants were analysed by cation exchange chromatography on a TSKgel SP-STAT column (4.6mm ID x 10cm L) featuring nonporous 7µm particles. The same column, sample and method were used to verify the column-system compatibility for both systems.

At first glance, the chromatograms (see Figure 1a) look quite similar. However, upon a closer look, the column evidently performs better in the UHPLC system than it does when connected to the HPLC system. The number of theoretical plates is six per cent higher for the UHPLC setup. The improvement is quite obvious when taking a look at the main peak (see Figure 1b). The peak width is smaller for the UHPLC chromatogram than for the HPLC chromatogram.

Gel Filtration and UHPLC

Protein aggregation is a common issue encountered during expression, purification and formulation of protein biotherapeutics. Aggregation needs to be characterised and controlled during the development and production of mAbs. Even small amounts of aggregates can alter the mAb therapy's function as an effective therapeutic.

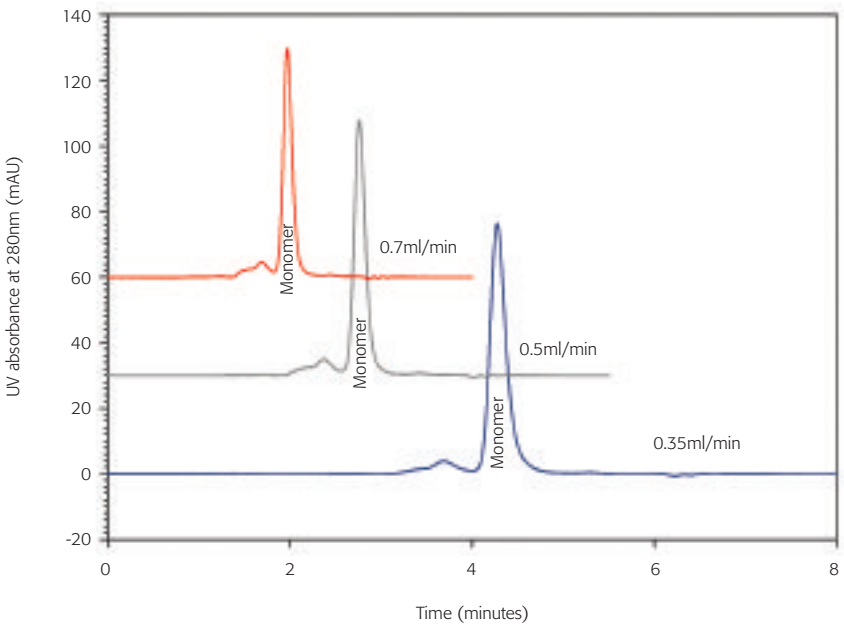

Figure 2: Aggregated mAb sample analysed on TSKgel SuperSW mAb HTP (4.6mmID x 15cm L) at three different flow rates (0.35mL/min; 0.5mL/min and 0.7mL/min). Mobile phase: 0.1M sodium phosphate, pH 6.7 + 0.1 M sodium sulfate; Detection: UV at 280nm; Injection volume: 5µL

High-performance size-exclusion liquid chromatography (SEC) on silica-based columns is the industry's standard technique for separating and quantifying soluble protein aggregates – an analysis which is always required for regulatory approval.

Using new column formats, perfectly adjusted to new technical developments, it is possible to considerably shorten analysis time or further increase resolution for aggregate determination. Figure 2 shows an example of what is possible with a short gel filtration column made of UHPLC column hardware and filled with 4µm particles and a UHPLC system.

Depending on the resolution required, it is possible to shorten analysis time for the antibody aggregates from 15 minutes for a conventional 30cm long column with 5µm particles down to four minutes when using a 15cm column with 4µm silica particles. This particle size is still large enough to keep the pressure in a moderate range, avoiding frictional heating inside the column, which could cause further aggregation or fragmentation during the process. These kinds of artefacts would imply a higher degree of impurities than actually exist within the sample vial.

Improving Detector Performance

Besides column performance and the choice of an appropriate method, the instrumentation is also crucial. A quite simple but effective improvement is to use optimised

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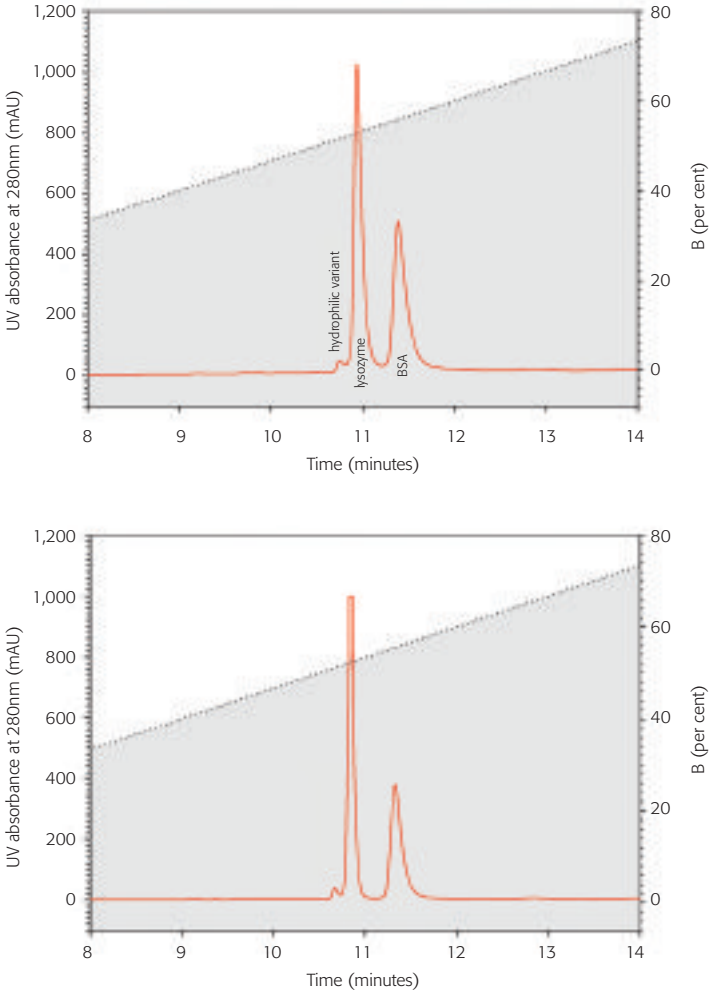

Figure 3a: Lysozyme from hen egg white and bovine serum albumin are separated on a TSKgel Protein C4-300 reversed phase column (2mmID x 5cm L). The 3µm particles offer resolution of the two proteins and a hydrophilic variant. The UHPLC UV detector was equipped with a 11µL standard flow cell; Flow rate: 0.2mL/min; Mobile phase A: 10/90/0.05 = ACN/H2O/TFA, B: 80/20/0.05 = ACN/H2O/TFA; Gradient: 0-100 per cent B in 15 minutes; Detection: UV at 215nm; Injection volume: 1µL; Sample: BSA & lysozyme (1µg/µL)

Figure 3b: Exchanging the UV flow cell to a semi-micro 2.5µl flow cell led to an increase in resolution of the two proteins by approximately 25 per cent when applying the same conditions

detector flow cells. Many instrument manufacturers offer different UV cell sizes. A standard UHPLC flow cell of a common UHPLC system has a volume of up to 11µL, whereas semi-micro flow cells feature volumes of 2-2.5µL.

These different types and volumes of flow cells are also common for HPLC systems. Figure 3a shows a chromatogram that was detected with a standard 11µL flow cell within a UHPLC system. The two standard proteins, lysozyme and BSA, are separated by reversed phase chromatography on a wide pore TSKgel Protein C4-300 (2mm ID x 5cm L). The two components can be resolved by a factor of 2.28. A significant increase of more than 25 per cent in resolution between the two peaks could be achieved by applying the 2.5µL detector flow cell to the same system and column, resulting in a resolution factor of 2.88 (see Figure 3b).

From that perspective, a UHPLC system does not necessarily outclass an up-to-date HPLC system; it might be worth putting the actual focus on the optimisation of the system, regardless of whether the client is working with an HPLC system or a UHPLC system.

Conclusion

Generally, there is no reason for not using HPLC columns in UHPLC systems. Ultimately, UHPLC and HPLC are still siblings of the same family of liquid chromatography. Bioseparation method transfer from HPLC to UHPLC is hardly more elaborate than from one HPLC instrument to another. In most cases, the applied pressure is far from exceeding the limit of a conventional HPLC system. This is the case in most bioseparation applications, since many biological sample molecules themselves cannot withstand very high pressure.

Transferring established HPLC methods using HPLC columns to a UHPLC system is an economical and time-saving way to take advantage of this latest wave of development in HPLC technology.

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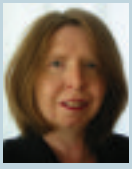

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