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The importance of system band broadening in modern size exclusion chromatography

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ABSTRACT

In the last few years, highly efficient UHP-SEC columns packed with sub–3 μm particles were commercialized by several providers. Besides the particle size reduction, the dimensions of modern SEC stationary phases (150 × 4.6 mm) was also modified compared to regular SEC columns (300 × 6 or 300 × 8 mm). Because the analytes are excluded from the pores in SEC, the retention factors are very low, ranging from $-1 < k < 0$, resulting in very small column band variance. Therefore, the contribution of the system itself to peak variance can become significant under UHP-SEC conditions.

The goal of this study was to evaluate the loss of efficiency observed with three different instruments (regular HPLC, non-optimized UHPLC and fully optimized UHPLC) offering different system variances. It appears that the new 150 × 4.6 mm, sub–3 μm SEC columns cannot be employed on a regular HPLC instrument, since the efficiency loss was equal to 60–85%, when analyzing mAb sample. Optimized UHPLC systems having very low extra-column volumes (typically $V_{ec} < 10$ μL) have therefore to be used to properly operate these columns. Due to the instrument contribution to band broadening, the apparent efficiency of SEC columns packed with sub–2 μm particles can indeed be hampered when using inappropriate system. Considering the extra-column band broadening contribution of current UHPLC instruments, a further decrease of SEC column dimension is therefore not desired.

1. Introduction

Size exclusion chromatography (SEC) is a traditional technique, widely applied for protein characterization and quality control. Its main application is the quantitative determination of protein aggregates [1–3].

In SEC, proteins are separated based on their difference in hydrodynamic volume in the mobile phase, by diffusion in and out of stagnant pores. Conventional SEC is typically performed using 6–8 mm ID × 300 mm columns packed with 5–10 μm particles and operating at moderate pressure (typically $\Delta P < 80$ bar) [4]. Ultra-high performance size-exclusion chromatography (UHP-SEC) columns offer new opportunities to improve both the resolution and throughput. UHP-SEC columns packed with 1.7, 1.8 or 2.0 μm particles with varying pore sizes of 125–900 Å are now available in 150 × 4.6 mm dimensions [5–9]. Very high pressures (up to 500 bar) can be generated when using such columns, leading to thermal effects and shear forces that might become critical for temperature- or pressure-sensitive proteins, with an additional risk of on-column protein denaturation and aggregation [10–12]. In the meantime, UHP-SEC was found to be suitable for the routine characterization of monoclonal antibodies (mAbs) [13], confirming that pressure-, temperature- and salt-induced denaturation are strongly protein dependent [14–17]. The pressure itself can also impact the retention (elution) time, based on the Gibbs free energy. However, ideally in SEC – due to the different elution mechanism compared to sorptive chromatography – the elution volume of proteins (and therefore selectivity) should not be affected by pressure [18].

In UHP-SEC, the throughput was found to be in average three-times higher, compared to conventional SEC methods [10,13] and the analysis time typically ranged between 3 and 8 min when using 150 × 4.6 mm columns. An important aspect to consider when using very efficient 150 × 4.6 mm SEC columns is the band dispersion due to the chromatographic system itself. As reported for reversed phase liquid chromatography (RPLC) separations, a suitable chromatographic system has to be employed to take the full benefits of small modern columns [19–22]. Indeed, in modern RPLC conditions, the column volume and related volumetric band variance are inherently low, due to the column dimensions (50 × 2.1 mm i.d. columns), and hence significantly affected by the extra-column band variance. In SEC, narrow bore columns are not

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Table 1
Detailed configuration of a (1) conventional HPLC (Waters Breeze), (2) a non-optimized UHPLC (first generation of Waters Acquity UPLC) and (3) optimized UHPLC (Waters Acquity UPLC I-Class) systems.

Instrument	inlet tubing i.d. (mm) x length (mm)	outlet tubing i.d. (mm) x length (mm)	flow cell volume (μL)	total extra-column volume (μL)	loop volume (μL)
Waters HPLC Breeze	0.125 × 600	0.125 × 700	10	65	100
Waters Acquity UPLC.	0.125 × 400	0.100 × 250 + 0.125 × 150	2 + 0.5	24	5
Waters Acquity UPLC I-Class	0.075 × 300	0.065 × 300	0.5	7	NA

available, but it is also important to keep in mind that band variance (σ^2_{col}) depends on the solute retention factor ($\sigma^2_{col} \sim (k+1)^2$) too. Since the compounds are excluded from the internal pores in SEC, the retention factor is very low (between $-1 < k < 0$) and so does the $(k+1)^2$, resulting in a small column band variance. As shown in [23] for polymers, the extra-column band broadening of regular HPLC system was still acceptable with conventional 300 × 6.8 mm SEC columns. Depending on the size of the solutes (that influences $(k+1)^2$, as large compounds have low values, since the largest compounds are more excluded and spend less time in the internal pores), extra-column band broadening represented between 2 and 13% of the column's intrinsic band broadening [23]. Extra-column band broadening in SEC has also been investigated in other studies and it has been concluded that this contribution can be kept sufficiently low in most practical situations, when using conventional SEC columns [3,23,24]. It has also been shown that the most important source of extra-column band broadening for the SEC separation of polymers was the tube dispersion [24].

However, problems are expected when SEC columns are miniaturized, without concomitant adaptation of the instrumentation. In UHP-SEC practice, extra-column band broadening probably impacts the observed column efficiency of the 150 × 4.6 mm SEC columns packed with sub–2 μm particles (due to the decreased volumetric band variance). The aim of this work was to evaluate the effect of extra-column dispersion on a (1) conventional HPLC, (2) a non-optimized UHPLC and (3) an optimized UHPLC systems, when operating 150 × 4.6 mm (1.8 μm) and 300 × 4.6 mm (3 μm) SEC columns. The extra-column volumes and variances of the three systems were thoroughly measured and the column's intrinsic efficiency was determined for a commercial therapeutic mAb (partially excluded) and for uridine (non-excluded, t_0 indicator). The apparent and remaining column efficiencies were evaluated. Finally, apparent plate heights (H) for systems possessing various extra-column variances were calculated for different column volumes.

2. Experimental

2.1. Chemicals and columns

Potassium chloride, potassium phosphate dibasic and hydrochloric acid were purchased from Sigma Aldrich (Buchs, Switzerland). Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). The aqueous mobile phase was used for one week.

Pertuzumab as therapeutic monoclonal antibody (mAb) was kindly provided by the Center of Immunology Pierre Fabre (Saint-Julien en Genevois, France), whereas blue dextran and uridine were purchased from Sigma Aldrich (Buchs, Switzerland).

The Yarra SEC-X150 (1.8 μm, 150 mm × 4.6 mm, 150 Å) and Yarra SEC-3000 (3.0 μm, 300 mm × 4.6 mm, 290 Å) columns from Phenomenex (Torrance, CA, USA) were selected, as they showed high column efficiency in previous study [10].

2.2. Equipment and software

Three different liquid chromatography (LC) systems from Waters were selected to study the extra-column band broadening: 1) Waters Breeze HPLC, 2) Waters Acquity UPLC (first generation) and 3) Waters Acquity UPLC I-Class (last generation UHPLC). These instruments were equipped with a binary delivery pump, an autosampler and ultraviolet detector (UV) operating at 220 nm. HPLC Breeze and Acquity UPLC used a loop offline injection device, while Acquity UPLC I-Class uses a flow through needle (FTN) injector. Acquity UPLC instrument was also supplied with a fluorescence detector (FL) in series before the UV detector. The non-optimised Acquity UPLC system represents an ideal compromise between the old HPLC Breeze system and the state-of-the-art Acquity UPLC I-Class system. Measured extra-column volumes and details of the three systems are listed in Table 1.

Data acquisition and instrument control were performed by Empower Pro 3 software (Waters). Calculations and data transferring were achieved by using Excel (Microsoft).

2.3. Chromatographic conditions and standard preparation

The mobile phase was composed of 200 mM potassium phosphate dibasic buffer and 100 mM potassium chloride in water, pH = 6.8 (adjusted with hydrochloric acid). The flow rate was varied by 50 μL/min steps from 50 μL/min to the maximum operating flow rate of the column (400 μL/min). Measurements were performed at a temperature of 25 °C.

The pertuzumab (IgG1 mAb) sample was diluted from the concentrated commercial solution (30 mg/mL) to 2.0 mg/mL in water, whereas uridine and blue dextran solutions were prepared from powder at 2.0 mg/mL in water. The injection volume was 1 μL, corresponding to 2 μg mass injected onto the column.

3. Methodology and calculations

When assuming that all contributions to peak variance are independent, and the peak has a Gaussian shape (distribution), the observed total variance (σ^2_{total}) of a peak is the sum of two contributions:

$$\sigma^2_{total} = \sigma^2_{col} + \sigma^2_{ec} \quad (1)$$

Where σ^2_{col} and σ^2_{ec} account for the column variance and the system variance, respectively. The extra-column band broadening (σ^2_{ec}) depends on the injected volume, the radius and length of connector tubing, the detector cell volume, the detector time constant, the flow rate, the diffusion coefficient of the sample and the mobile phase composition [20].

The purpose of this study was to determine the overall σ^2_{ec} of our instruments. Typically, the overall extra-column variance is measured by leaving out the chromatographic column, directly connecting the inlet to the outlet tubing using a zero dead-volume (ZDV) union [25]. Obviously, this way σ^2_{ec} is measured at lower pressure compared to the case when operating the column, and therefore the viscosity and diffusion coefficients can be different.