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Estimation of pressure-, temperature- and frictional heating-related effects on proteins' retention under ultra-high-pressure liquid chromatographic conditions

Szabolcs Fekete*, Davy Guillarme

School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Bd d'Yvoy 20, 1211 Geneva 4, Switzerland

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ABSTRACT

The goal of this work was to evaluate the changes in retention induced by frictional heating, pressure and temperature under ultra high pressure liquid chromatography (UHPLC) conditions, for four model proteins (i.e. lysozyme, myoglobin, fligrastim and interferon alpha-2A) possessing molecular weights between 14 and 20 kDa. First of all, because the decrease of the molar volume upon adsorption onto a hydrophobic surface was more pronounced for large molecules such as proteins, the impact of pressure appears to overcome the frictional heating effects. Nevertheless, we have also demonstrated that the retention decrease due to frictional heating was not negligible with such large biomolecules in the variable inlet pressure mode. Secondly, it is clearly shown that the modification of retention under various pressure and temperature conditions cannot be explained solely by the frictional heating and pressure effects. Indeed, some very uncommon van't Hoff plots (concave plots with a maximum) were recorded for our model/therapeutic proteins. These maximum retention factors values on the van't Hoff plots indicate a probable change of secondary structure/conformation with pressure and temperature. Based on these observations, it seems that the combination of pressure and temperature causes the protein denaturation and this folding-unfolding procedure is clearly protein dependent.

1. Introduction

Numerous studies illustrated that the analyte retention can significantly be altered by pressure-related events, even in conventional high-performance liquid chromatography (HPLC) pressure range (≤400 bar), [1–6]. Obviously, these effects are much more relevant in ultra-high pressure LC (UHPLC) conditions [7–11]. When working in very high pressure conditions (e.g. 800–1000 bar), two main contributions can be observed: (1) the effect of pressure itself and (2) the effect of temperature gradients caused by frictional heating, which becomes predominant when working at high linear velocities [12].

The shift in retention (k) caused by pressure can be mostly explained by the change of molar volume and its dependence on the pressure (P) is often derived from the Gibbs free energy equation [13]. Several studies have demonstrated that k increases with pressure [14,15]. For low-molecular-weight analytes the retention increase is moderate, while the increase has been found to be more pronounced for large analytes (e.g. peptides and proteins) [16–18]. Pressure impacts not only the molar volume but can also have a strong influence on the solvation layer of an alkyl-bonded phase; on the solvation shell of the hydrophobic regions of the analytes; and on the hydration shell of the hydrophilic parts [16]. A reduced solvation layer increases molecule hydrophobicity, and therefore increases k under reversed-phase conditions. Pressure can also have an impact on other intrinsic parameters of a chromatographic separation, such as column void volume or intrinsic column porosity and therefore on the phase ratio [19,20]. Other studies have shown that the variation of retention with pressure could be related to changes in mobile phase pH and to the extent of analyte charges [21]. A recent study showed that pressure-induced change in retention was temperature dependent [13], while it was assumed to be independent. The impact of pressure was found to be less important at elevated temperature for both small analytes and peptides. A model was proposed to calculate the expected increase in retention with pressure and temperature [13].

For large molecules such as proteins, it has been demonstrated that pressure has a rather strong influence on retention, even in gradient elution mode [18]. Important modification of retention and slight changes in selectivity and resolution were reported [18]. This

* Corresponding author. Tel.: +36 30 395 6657.
E-mail address: szabolcs.fekete@unige.ch (S. Fekete).

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study also demonstrated that pressure-induced conformational changes of proteins were highly probable under RP conditions. As mentioned, the change in molar volume caused by the pressure is a suitable parameter to model the effect of pressure on solute retention. However, molar volume is complex as it can be correlated with several associated phenomena taking place during the adsorption process. It should be expected, especially in the case of macromolecules that changes in molar volume may originate from different sources, such as the variations in the energy of molecular interactions, solvation, aggregation or changes in the energy density of these interactions [22]. These effects probably play a key role in the retention of proteins. Conformational changes induced by pressure, besides affecting directly molar volume, can also modify the surface hydrophobicity of the molecule [16]. The conformational change (folding or unfolding) of a protein molecule upon adsorption is a well-known phenomenon that leads to the exposure of its hydrophobic core. At higher pressures, the adsorption of proteins onto the stationary phase could therefore be more pronounced.

To evaluate the sole effect of pressure on analyte retention, data are generally gathered by adding restriction capillary at the column outlet, to increase pressure (decrease apparent permeability), while avoiding frictional heating effects related to high mobile phase linear velocity [13,18,21]. This way, the retention data observed at a given flow-rate but at different inlet pressures can easily be compared.

When working at high pressure, important frictional heating can be developed at elevated mobile phase linear velocities. The generated heat power (P_f) is the product of linear velocity (u) and pressure drop along the column (L). The generated heat leads to several effects (especially when poorly dissipated, e.g. still-air ovens) and finally the temperature inside the column increases and forms both radial and longitudinal temperature gradients [15]. These temperature gradients affect both the analyte retention and band broadening. The effects of radial temperature gradients on the efficiency (band broadening) are well documented [23–26]. However, the impact of longitudinal temperature gradients on solute retention is not simply measurable, as in many cases, the effect of both pressure and temperature gradients occur at the same time and are hard to dissociate. A recent study demonstrated the experimental dissociation of longitudinal temperature gradients from other pressure-related effects [12]. For this purpose, the flow-rate was systematically varied while the inlet pressure was kept constant at 800 bar by adding different restrictor capillaries to the column outlet. It was proved that the longitudinal temperature gradient caused by frictional heating is less important for large molecules than for small analytes, but its existence was measurable even for insulin of ~5.8 kDa. In the constant inlet pressure mode, a slight decrease in insulin retention was observed with increasing flow-rate, while in variable pressure mode the opposite trend was highlighted.

Temperature-related retention changes are generally described by the van't Hoff relationship. With large biomolecules, the effect of temperature on retention may become more complex. Indeed, depending on the stability of the secondary structure, the molecules unfold to various extents and hence interact with the stationary phase with various strengths [27]. Due to the different conformation-dependent responses of proteins at elevated temperatures, the change in retention can be very different from one protein to the other one [28,29]. Therefore, temperature appears as an interesting parameter to tune selectivity. Under certain conditions, the native conformation and/or other intermediate conformations may be present during the analysis. Each of these will interact differently with the stationary phase, resulting in varying retention times or multiple peaks observed in the chromatogram [30–36]. In some cases, irreversible conformational changes can occur by changing the temperature. Irreversible temperature-induced conformational transitions may have been responsible for observed peak splitting of proteins in RPLC conditions [37]. For peptides—similar to small analytes—a retention decrease is often observed at elevated temperature [27]. In contrast, for insulin, lysozyme and RNase, retention increase was observed when modifying the temperature from 25 to 50 °C (insulin) and 25 to 40 °C (lysozyme, RNase) [16,38].

Up to now, the effect of longitudinal temperature gradients—dissociated from pressure—on the retention of proteins has not been published. Moreover, pressure- and temperature-related retention changes of proteins under ultra-high pressure conditions (P > 400 bar) have not been reported yet. In this study, the effects of frictional heating and pressure on the retention of proteins were experimentally dissociated for the first time.

The aim of this study was to estimate the changes in retention induced by (1) frictional heating, (2) pressure and (3) temperature for moderate size model proteins possessing molecular weights between 14 and 20 kDa. The experiments were performed using restrictor tubing, to evaluate the pure effect of frictional heating, while neglecting the effect of pressure. The experimental work was conducted at up to ~750 bar inlet column pressure (excluding system pressure). On the other hand, the cumulative effect of pressure and frictional heating was studied at various temperatures in the range of 25–75 °C. Systematic experiments were performed with a 50 × 2.1 mm column packed with state-of-the-art fully porous wide-pore sub-2 μm particles.

2. Experimental

2.1. Chemicals, columns

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). Acetonitrile (gradient grade) was purchased from Sigma-Aldrich (Buchs, Switzerland). Model standard proteins such as lysozyme (from chicken egg white, MW ~ 14.7 kDa) and myoglobin (from equine skeletal muscle, MW ~ 17.6 kDa) were purchased from Sigma-Aldrich. Recombinant human granulocyte-colony stimulating factor (G-CSF or filgrastim, MW ~ 18.8 kDa) was obtained from Amgen (Switzerland). Recombinant interferon alfa-2A (MW ~ 19.2 kDa, Roferon) was obtained from Roche Pharma (Switzerland). Trifluoroacetic acid (TFA) and uracil were also purchased from Sigma-Aldrich.

Waters Acquity BEH300 C4 (50 × 2.1 mm) column packed with 1.7 μm particles was purchased from Waters (Milford, MA, USA).

2.2. Equipment, software

All the measurements were performed using a Waters Acquity UPLC™ I-Class system equipped with a binary solvent delivery pump, an autosampler and an UV detector. The system includes a flow through needle (FTN) injection system with 15 μl needle and a 0.5 μl UV flow-cell. The connection tube between the injector and column inlet was 0.003″ (0.08 mm) I.D. and 200 mm long (active preheating included), while the capillary located between the column and detector was 0.004″ (0.10 mm) I.D. and 200 mm long. The overall extra-column volume (V_{ext}) was about 8.5 μl as measured from the injection seat of the auto-sampler to the detector cell. The average extra-column peak variance of our system was found to be around σ^2_{ec} ~ 0.5–3 μl^2 (depending on the flow-rate, injected volume, mobile phase composition and solute). Data acquisition and instrument control were performed by Empower Pro 2 Software from Waters.

Column pressure was increased by connecting in series at the column outlet, one or several capillary tubes of 50 μm I.D. and lengths of 10, 20 and 50 cm (it was possible to couple up to 100 cm