Journal of Chromatography A, 1311 (2013) 65–71

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Influence of pressure and temperature on molar volume and retention properties of peptides in ultra-high pressure liquid chromatography

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ARTICLE INFO

Article history:
Received 24 April 2013
Received in revised form 12 July 2013
Accepted 12 August 2013
Available online 18 August 2013

Keywords:
UHPLC
Ultra-high pressure
Retention
Molar volume
Pressure effect
Peptides

ABSTRACT

In this study, pressure induced changes in retention were measured for model peptides possessing molecular weights between ~1 and ~4 kDa. The goal of the present work was to evaluate if such changes were only attributed to the variation of molar volume and if they could be estimated prior to the experiments, using theoretical models. Restrictor tubing was employed to generate pressures up to 1000 bar and experiments were conducted for mobile phase temperatures comprised between 30 and 80 °C. As expected, the retention increases significantly with pressure, up to 200% for glucagon at around 1000 bar compared to ~100 bar. The obtained data were fitted with a theoretical model and the determination coefficients were excellent ($r^2 > 0.9992$) for the peptides at various temperatures. On the other hand, the pressure induced change in retention was found to be temperature dependent and was more pronounced at 30 °C vs. 60 or 80 °C. Finally, using the proposed model, it was possible to easily estimate the pressure induced increase in retention for any peptide and mobile phase temperature. This allows to easily estimating the expected change in retention, when increasing the column length under UHPLC conditions.

1. Introduction

Liquid solutions are normally considered to be incompressible, however several studies showed that in conventional HPLC pressure ranges (<400 bar), solute retention can be significantly altered [1–6]. Obviously, the pressure effects are much more important in ultra-high pressure LC (UHPLC) conditions [7–11]. In order to study the pure effect of pressure, data are generally gathered using a restriction capillary at the column outlet to increase pressure, while avoiding frictional heating effects related to high mobile phase flow rates. Generally, pressure influences the mobile phase density, viscosity and temperature, the analytes' diffusion rate and the strength of interactions between solute, stationary phase and mobile phase [12]. Therefore, the pressure in LC can significantly influence the retention (k) of any type of analytes.

First, Giddings showed that pressure could induce important changes in molecular volume and also alter the ability of molecules to crowd together, to reduce molecular volume [13,14]. Numerous theoretical aspects of the influence of pressure on the chromatographic process have been explained and described by Martin and Guiochon [15]. Several publications have shown that k increases with a pressure enhancement [16,17]. While the increase for low-molecular-weight analytes is relatively modest, the increase for large analytes (e.g. peptides, proteins) has been found to be much larger [18–20]. This effect is primarily related to the changes in molecular molar volume (V_m). However, pressure also has a strong influence on the solvation layer of the alkyl-bonded phase; on the solvation shell of the hydrophobic regions of the protein; and on the hydration shell of the hydrophilic parts [18]. A reduced solvation layer increases molecule hydrophobicity, and therefore increases k under reversed-phase conditions. Pressure can also affect other intrinsic parameters of chromatographic separation, such as column void volume and intrinsic column porosity [15,21].

The effects of pressure on thermodynamic equilibrium were studied and it was reported that changes in column pressure could produce equilibrium changes in the distribution of the analyte between stationary and mobile phases. The magnitude of these changes depends on the analyte–solvent interconnection and on the changes in solvent structure within the mobile and stationary phase [7,22,23,13]. It was also demonstrated that analyte molecular volume changes under elevated pressure were among the major variables affecting analyte retention [13]. Other studies have shown that the variation of retention with pressure can be related to changes in mobile phase pH and to the extent of analyte ion-dissociation [24].

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http://dx.doi.org/10.1016/j.chroma.2013.08.045

Numerous works have demonstrated the importance of pressure on the analyte retention for small molecular weight compounds. Under conventional HPLC conditions, an increase in retention factors between 9 and 24% was observed for fatty acids when increasing the pressure from 100 to 350 bar [5,6]. An important change in retention and selectivity of weak acids and bases, and even a change in elution order were also demonstrated [24]. Another study showed retention changes between −7 and +12% under conventional HPLC conditions as well [3]. With short, narrow bore columns packed with conventional 5 μm particles, 2 to 12% increase in retention was reported for neutral solutes, while the most important changes (35–50%) were observed for acidic and basic analytes [7,8]. The changes in retention were significantly larger (by a factor of 3) when ion-pairing reagent was added to the mobile phase in RPLC mode [25]. Finally, the effect of pressure was also demonstrated in normal phase mode and chiral separations [2,26].

For large molecules such as peptides and proteins, it has been observed that pressure can have a rather strong influence on retention [18–20]. Even in gradient elution mode, important changes in retention and slight changes in selectivity and resolution were reported [20]. A recent study demonstrated that pressure induced conformational changes of proteins are highly probable under RP conditions [20]. As mentioned, the change in V_m caused by the pressure is a suitable parameter to model the effect of pressure on solute retention. However, ΔV_m is complex as it can be correlated with several associated phenomena taking place during the adsorption process [18]. It should be expected, especially in the case of macromolecules that changes in V_m 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 [27]. These effects probably play a key role in the retention of peptides or proteins. Conformational changes induced by pressure, besides affecting V_m directly, can also modify the surface hydrophobicity of the molecule [18]. 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. Therefore, modelling the pressure effects on retention could become very complex for proteins.

The goal of this study was to experimentally measure the pressure induced changes in retention for model peptides possessing molecular weights between ~1 and ~4 kDa. These experiments were performed using restrictor tubing, to evaluate the pure effect of pressure, while neglecting as much as possible the influence of frictional heating. Finally, the experimental work was conducted for pressures up to ~1000 bar and for various mobile phase temperatures comprised between 30 and 80 °C. The experimental data were then fitted with theoretical models, to evaluate if theory was able to predict pressure induced changes in retention, only taking into account the variation of molar volume.

2. Theory

The dependence of the retention factor, k, of a compound on the pressure, p, can be derived from Gibbs free energy as follows:

$$\ln k = -\frac{\Delta G}{RT} + \ln\phi = -\frac{\Delta E}{RT} - p\frac{\Delta V_m}{RT} + \frac{\Delta S}{R} + \ln\phi \quad (1)$$

where ΔG is the change of Gibbs free energy, ΔE the change of internal energy of the system, ΔV_m the change of molar volume of solute during adsorption, ΔS the change of system entropy, ϕ the phase ratio, R the universal gas constant, and T the absolute temperature.

By rearranging Eq. (1), k can be calculated at any pressure as:

$$k = k_0 \exp\left(-\frac{\Delta V_m}{RT}p\right) \quad (2)$$

where k_0 is the limiting value of the retention factor at zero pressure:

$$k_0 = \phi \exp\left(-\frac{\Delta E}{RT} + \frac{\Delta S}{R}\right) \quad (3)$$

Eqs. (2) and (3) cannot be used directly for the determination of the compound retention time since the retention factor changes gradually during the compound migration, due to the pressure gradient inside the column. In the case of isocratic elution, a linear pressure gradient can be assumed. In this case, the local pressure at any position, z, in the column is given as:

$$p[z] = p_{in} - \frac{\Delta p}{L}z \quad (4)$$

where p_{in} is the inlet pressure (pressure at the head of column), and Δp the pressure drop across the column. Note, that Eq. (4) is valid only in case of isocratic elution assuming that the phase ratio of the column is constant throughout the column. In our case, the change of phase ratio was negligible (see Section 3 for more details).

By combining Eqs. (2)–(4), the local value of the compound retention factor can be written as:

$$k[z] = k_0 \exp\left[-\frac{\Delta V_m}{RT}\left(p_{in} - \frac{\Delta p}{L}z\right)\right] = k_{in}\exp\left(\frac{\Delta V_m}{RT}\frac{\Delta p}{L}z\right) \quad (5)$$

where k_{in} is the retention factor of solute at the head of column (at the inlet pressure, p_{in}).

The local migration velocity of the zone of a compound at any position, z, in the column can be calculated with the knowledge of $k[z]$.

$$\frac{dz}{dt} = \frac{u_0}{(1+k[z])} \quad (6)$$

where u_0 is the linear velocity of the eluent ($u_0 = L/t_0$, where t_0 is the hold-up time of the column). Accordingly, the retention time, t_R, can be calculated by integrating Eq. (8).

$$t_R = \int_0^{t_R} dt = \frac{1}{u_0}\int_0^L (1+k[z])dz \quad (7)$$

The integration can be accomplished by substituting Eq. (5) in Eq. (7). Accordingly,

$$t_R = \frac{1}{u_0}\int_0^L \left(1 + k_0\exp\left[-\frac{\Delta V_m}{RT}\left(p_{in} - \frac{\Delta p}{L}z\right)\right]\right)dz \quad (8)$$

and

$$t_R = t_0\left[1 + \frac{k_0 RT}{\Delta p\,\Delta V_m}\exp\left[-\frac{p_{in}\,\Delta V_m}{RT}\right]\left(\exp\left[\frac{\Delta p\,\Delta V_m}{RT}\right] - 1\right)\right] \quad (9)$$

Eq. (9) can be used for the migration time calculation of any compounds through the separator column with the knowledge of operating parameters of the chromatographic system and fundamental molecular and thermodynamic properties.

3. Experimental

3.1. Chemicals, columns

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). Acetonitrile and Methanol (gradient