Mobile Phase Viscosity and Velocity Dependence on Protein Retention Using Nonequilibrium Chromatographic Techniques

Yves Claude Guillaume,* Mireille Thomassin, and **Christiane Guinchard**

Equipe de Chimie Therapeutique, Laboratoire de Chimie Analytique, Faculte de Medecine Pharmacie, Place Saint Jacques, 25030 Besancon Cedex, France

Abstract

Nonequilibrium chromatography (NEC) is an alternative chromatographic procedure for the separation of macromolecules. The retardation of a protein series is studied using a phosphate buffer as a mobile phase with various concentrations of glycerol fraction (used as a viscosity modifier) at different mobile phase velocities and a C1 column with a very low packing particle diameter as a stationary phase. It is shown that the two factors (viscosity and velocity) of the mobile phase constituted important parameters in the retention mechanism of the proteins in NEC. The retardation velocity domain is divided into two regions. For low velocity regions, the protein retention decreased with a mobile phase velocity increase. This retention is enhanced above a critical value of the mobile phase velocity. The transition between the two well-known NEC methods, slalom chromatography and hydrodynamic chromatography, is clearly visualized for the first time for the protein retention of particular values of the mobile phase velocity.

Introduction

The separation of biomolecules by conventional high-performance liquid chromatographic (HPLC) modes is usually based on an equilibrium phenomenon between mobile and stationary phases. Alternative chromatographic procedures are available for biological and synthetical polymers. The two techniques, slalom chromatography (SC) and hydrodynamic chromatography (HDC), are based on the use of the laminar flow, which occurs in the interstitial spaces created between the particles in the column (1–12). The separation process depends on the flow rate (F) and the particle size of the column packing and not on their pore size or chemical nature. HDC has been principally developed and applied to the separation of synthetic polymers such as polystyrenes (4–6). The elution order in HDC is the same as in gel-permeation chromatography (GPC) because of the exclusion for the large polymers from the low velocity regions near the particle wall (4). The separation is only valid when the polymer is in a random coil form (4). We have recently demonstrated the connection between SC and GPC processes (13). Separation in SC has been reported for double-stranded DNA molecules (6–11). The elution order for the DNA molecules is the opposite to that expected for an HDC or GPC mechanism, the larger strands are eluted after the smaller ones (6). When the DNA chain is applied to a chromatographic system, it frequently goes around the spherical obstacles; the larger the fragments, the more difficulty it has in traveling across the interstitial spaces created inside the column. We have also demonstrated that the SC and HDC modes are interconnected and that the HDC ↔ SC transition exists and is clearly visualized for the DNA fragments (14). Therefore, GPC, HDC, and SC modes can be linked in a global separation mechanism based on a nonequilibrium chromatographic (NEC) principle. In order to gain further insight into this fractionation mechanism, the retention of a protein series in a packed column with particles having a very low diameter was analyzed over a wide range of mobile phase F values and at various glycerol fractions used as a viscosity modifier.

Experimental

Apparatus

The HPLC system consisted of a Merck (Nogent Sur Marne, France) Hitachi pump, an Interchim (Montlucon, France) Rheodyne injection valve Model 7125 fitted with a microliter sample loop, and a Merck L4500 diode-array detector. A C1 column (2-µm particle size, 50- × 4.6-mm column size) supplied by Interchim was used with the controlled temperature at 25°C in an Interchim Crococil oven TMN°701. An RM 180 Rheomat temperature-stabilized rotary viscosimeter (Rheometric Scientific, Caluire, France) was used to measure the viscosity of each mobile phase (η).

* Author to whom correspondence should be addressed.

Reagents

Human serum albumin (HSA), bovine serum albumin (BSA), and chicken serum albumin (CSA) were supplied by Sigma Aldrich (Paris, France). Glycerol, sodium hydrogenphosphate, and sodium dihydrogenphosphate were purchased from Prolabo (Paris, France). Water was obtained from an Elgastat option water purification system (Odil, Talan, France) fitted with a reverse-osmosis cartridge.

Chromatographic conditions

The mobile phase consisted of a 0.001M sodium phosphate salt at pH = 6.5 with different fractions (m/m) of glycerol in order to modify η. The following ten glycerol fractions were included in this range: 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.10. Twenty microliters of protein solution were injected and the retention time measured for different F values varying from 0.02 to 1.8 mL/min.

Results and Discussion

NEC using reversed-phase columns

We have previously shown that columns developed for reversed-phase chromatography (such as the C1 column used in this study) with very low particle diameter are suitable for NEC (11). In order to specifically study the glycerol effect on η, recovery of the proteins was calculated from the chromatographic peak areas. The protein recovery at a constant F was globally identical (difference < 10%) to whatever was the value of the glycerol fraction in the eluent. Therefore, it can be concluded (15) that the glycerol effect on the relative retention time (RRT) values of the proteins was the result of a change in η. This also showed that the destabilizing effect of glycerol on proteins related to its capacity to interact with its reaction sites did not significantly affect the protein integrity over the glycerol concentration range studied.

Mobile phase velocity dependence on retention in NEC

In NEC, using the retention time (t_R) of the various protein species and the void time (t_o), the apparent RRT equal to t_R/t_o was used as a protein retention marker (14). The t_o value was obtained by a triplicate 1-µL injection of KI (i.e., the dead-time marker) (14). From the t_R and t_o values, the experimental RRT was calculated for the different chromatographic conditions. All of the experiments were repeated three times. The variation coefficients of the RRT values were less than 3% in most cases, indicating a high reproducibility and good stability for the chromatographic system. The RRT values were plotted against the F value for a given protein. Figure 1 shows the curve for HSA determining the F dependence on RRT for an eluent without glycerol. Beyond 1 mL/min, HSA protein was practically fragmented through the column. The plot was divided into two regions, one for F values below the critical value of the mobile phase velocity (F_c) and a second for values above F_c. It can be noted that in these

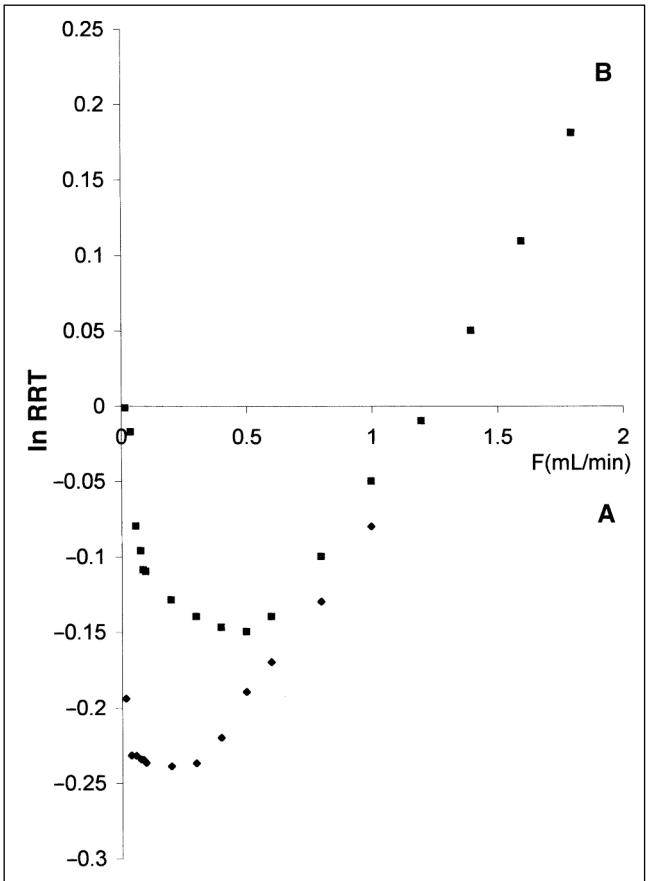

Figure 1. The HSA RRT in relation to the mobile phase F value (A) without glycerol (η = 0.92) and (B) for a glycerol fraction equal to 0.10 (η = 1.17).

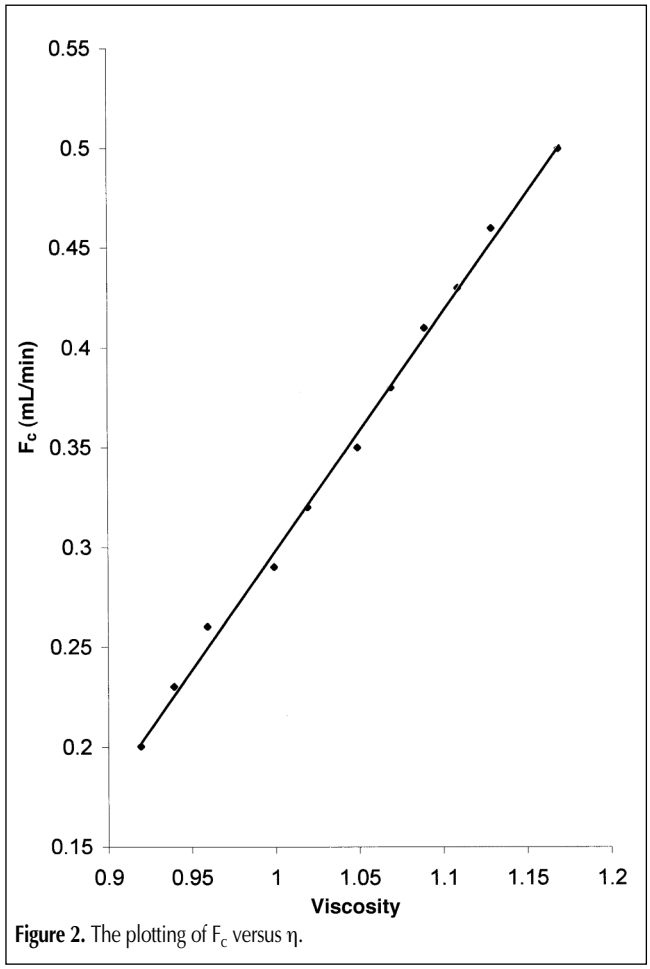

Figure 2. The plotting of F_c versus η.

two domains the log natural value of RRT that was less than 0 showed that the protein structure was in a random coil conformation (compact structure) (14).

When $F < F_c \cong 0.2$ mL/min, ln RRT decreased as F increased. In this very low F region, the random coil form remained relatively constant in the laminar flow occurring in the interstitial space between the particle packing in the column. When the mobile phase velocity increased, the proteins were excluded from the lowest velocity regions near the particle wall, producing a decrease in the RRT values (hydrodynamic retention behavior, HDR) (4).

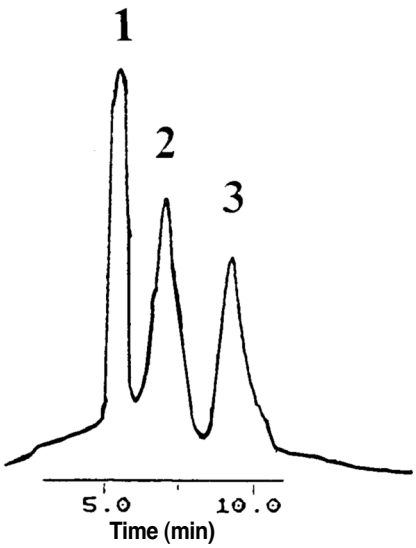

Figure 3. Chromatogram of HSA, 1; BSA, 2; and CSA, 3, at F = 0.06 mL/min for η = 1.17.

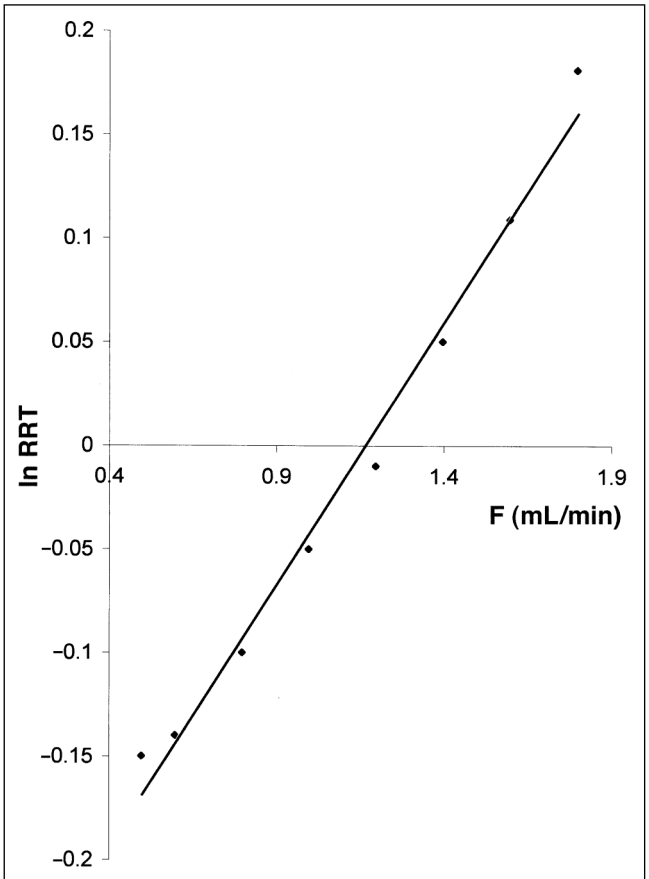

Figure 4. The HSA RRT in relation to the mobile phase F value in the range of 0.5 to 1.8 mL/min for a glycerol fraction equal to 0.10 (η = 1.17).

When $F > F_c$, a net increase in ln RRT values was surprisingly observed with an increase in F values. With the NEC technique, it is well-known that the macromolecule retention in the packed column is based on the effective radius of the random coil macromolecule (4). In this second domain contrary to the previous region ($F < F_c$), when F increased, the polymer stretching caused by the shear deformation resulted in a decrease in the size transverse in relation to the flow direction, making the effective radius of the random coil macromolecule decrease. Therefore, the proteins tended to go to the lowest velocity regions near the particle wall and the RRT value increased, thus confirming an HDR behavior.

Viscosity dependence on retention in NEC

For each glycerol fraction, the ln RRT versus F curve was similar to the previous ones (i.e., a critical F_c value was observed). Figure 1 shows the curve for HSA determining the F dependence on RRT for a glycerol fraction equal to 0.10 corresponding to η = 1.17. For this viscosity value, the HSA protein was practically fragmented through the column beyond 1.8 mL/min. A similar behavior was observed for the two other proteins, and the η values corresponded to each glycerol fraction in the mobile phase. A linear F_c versus η behavior was observed ($F_c = 1.19\eta - 0.90$, $r^2 = 0.997$) (Figure 2). The values and Figures 1 and 2 lead to the following conclusions.

In both $F < F_c$ and $F > F_c$ in which ln RRT < 0 (Figure 1), the ln RRT values increased as η increased. This phenomenon can be explained by the fact that when η increased there was a decrease in the effective radius of the random coil macromolecule. Therefore, the protein tended to go to the lowest velocity regions near the particle wall and the ln RRT value increased (HDR behavior). Figure 3 shows the chromatogram for the separation of the three proteins analyzed at 0.06 mL/min for η = 1.17.

The ln RRT became positive for η = 1.17, for example, when the particular value of F_p was above 1.2 mL/min (Figure 1). In this case, the protein structure began to lose its compact structure and it migrated through the packing more or less in a curvy fashion (unlike a spherical particle) via the SC mode. For η = 1.17 in the 0.5–1.8-mL/min F range, ln RRT versus F always had a linear behavior (Figure 1), resembling the equation ln RRT = 0.25F – 0.29 ($r^2 = 0.987$ for HSA, Figure 4). The F_p value, in which ln RRT changed its sign, corresponded to the HDC → SC transition. This result showed for the first time that the SC and HDC mechanisms for proteins can be interconnected, as was previously demonstrated for more flexible molecules such as a DNA fragment using the NEC technique (15,16).

Conclusion

In this study, it was demonstrated by using NEC techniques that the retention mechanism is strongly dependent on the pro-

tein conformation in the interstitial space created in the column. The hydrodynamic chromatographic mode was obtained particularly at a low F value. The beginning of the SC mode, often described for macromolecules that are more flexible than proteins, was also clearly visualized. This confirmed that SC and HDC can be interconnected even for less extensible macromolecules than DNA fragments classically used in NEC methods.

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