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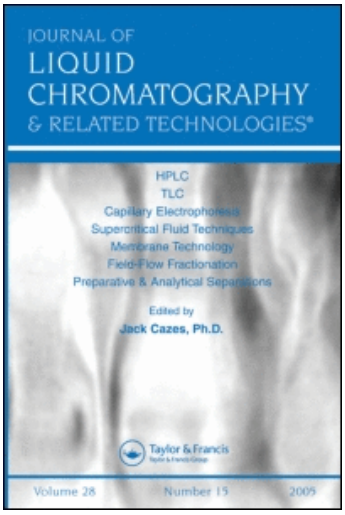

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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FLOW RATE DEPENDENCE ON THE BIOPOLYMER RETENTION IN HYDRODYNAMIC CHROMATOGRAPHY. COMPARISON BETWEEN THE BEHAVIORS OF PROTEINS AND PLASMIDS

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Online publication date: 31 May 2001

To cite this Article Peyrin, Eric , Guillaume, Yves C. , Villet, Annick , Ravel, Anne , Grosset, Catherine , Alary, Josette and Favier, Alain(2001) 'FLOW RATE DEPENDENCE ON THE BIOPOLYMER RETENTION IN HYDRODYNAMIC CHROMATOGRAPHY. COMPARISON BETWEEN THE BEHAVIORS OF PROTEINS AND PLASMIDS', Journal of Liquid Chromatography & Related Technologies, 24: 9, 1245 — 1252
To link to this Article: DOI: 10.1081/JLC-100103445
URL: http://dx.doi.org/10.1081/JLC-100103445

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J. LIQ. CHROM. & REL. TECHNOL., 24(9), 1245–1252 (2001)

FLOW RATE DEPENDENCE ON THE BIOPOLYMER RETENTION IN HYDRODYNAMIC CHROMATOGRAPHY. COMPARISON BETWEEN THE BEHAVIORS OF PROTEINS AND PLASMIDS

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ABSTRACT

Hydrodynamic chromatography (HDC) in a packed column is a useful chromatographic mode for the rapid separation of polymers. This paper compared the retention of various circular double-stranded DNA molecules (3, 5 and 10 kbp) and proteins (keyhole lumpet hemocyanin, ferritin, thyroglobulin, and catalase) in a chromatographic system using a C1 stationary phase and a acetonitrile-phosphate buffer mixture as a mobile phase. For a simi-

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lar molecular weight, the protein was retained more than the corresponding plasmid. This was attributed to the difference in the compactness between the two species. As well, the retention dependence on the flow rate (0.03 to 1.5 mL/min) exhibited a different behavior in relation to the type of biopolymer.

The protein retention curve showed a decrease in the relative retention time until 0.2 mL/min, followed by an increase for the high size polymers, only, while the plasmid retention increased over the entire flow rate range. This observation confirmed that the migration in HDC was dependent on two antagonist phenomena, i. e. stress induced diffusion and polymer deformation.

This work demonstrated that the HDC separation of protein was optimal for a flow rate equal to 0.2 mL/min.

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INTRODUCTION

The separation of molecules by high performance liquid chromatography is classically based on an equilibrium phenomenon between the eluent and the stationary phase.[1-8] However, alternative chromatographic procedures, based on a "non equilibrium principle,[9]" are available for the separation of biological or synthetic polymers. These consist in slalom chromatography (SC)[10-13] and hydrodynamic chromatography (HDC). HDC in packed columns has been developed and applied to the separation of various polymers, such as polystyrenes or polybutadienes.[14-16]

The elution order in HDC is the same as in size exclusion chromatography (SEC), due to the exclusion for the large polymers from the low velocity regions near the particle wall.[14] The macromolecules can be separated in packed columns on the basis of the effective radius of the random coil polymer r_{eff}.[14]

The separation in HDC occurs only when the ratio λ between r_{eff} and the effective channel radius (dependent on the particle diameter) is included between 0.01 and 0.35. This λ value is related to the relative migration parameter τ in such a way that a universal calibration for HDC can be obtained.[14]

As well, Poppe's group has studied the effect of the flow rate on the retention of synthetic polymers in packed columns.[16] However, to the best of our knowledge, no study has been carried out on the flow rate dependence on the HDC retention of proteins and plasmids in packed columns. In this paper, the retention of various circular DNA and proteins were studied on a C1 porous stationary phase over a wide range of flow rates and their elastic properties were compared. As well, the optimal conditions for the protein separation were obtained.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Varian pump 9010 (Les Ulis, France), a Jasco autosampler AS-950, a Varian 9050 UV-VIS detector (λ = 260nm) and a D-2500 chromato-integrator (Merck, Nogent-sur-Marne, France). The C1 Kromasil column (particle size: 5 μm, column size: 150 × 4.6mm, pore diameter: 100Å) was supplied by Interchim (Montlucon, France).

Reagents

The pBluescript II SK+ (2.96kbp, Mw = 1973 kDa) plasmid was purchased from Stratagene Europe (Amsterdam, the Netherlands). The pSG-CCG1 (10.6 kbp, Mw = 7066 kDa) plasmid was the coding sequence of CCG1/hTAF$_{II}$250 inserted in pSG5 (Stratagene Europe). The pSG-TBP (5.1kbp, Mw = 3400 kDa) plasmid was obtained as previously described.[18] Keyhole lumpet haemocyanin (Mw = 6000 kDa) Ferritin (Mw = 900 kDa), Thyroglobulin (Mw = 660 kDa), and Catalase (Mw = 250 kDa) were supplied by Calbiochem or Fluka. Potassium iodide, EDTA, sodium hydrogen phosphate, and sodium dihydrogen phosphate were purchased from Merck and Carlo Erba Reactifs (Val de Reuil, France). Acetonitrile HPLC was supplied by sds (Peypin, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France), fitted with a reverse osmosis cartridge.

Chromatographic Conditions

The mobile phase consisted of a sodium phosphate buffer 0.01 M-EDTA 1mM pH=6.8-acetonitrile 80/20 (v/v) mixture. The flow rates varied from 0.03 to 1.5 mL/min. 20 ng of the DNA or protein solutions were injected in triplicate and the retention times were measured at the different flow rate values. The respective void time was obtained by the injection, in triplicate, of 1 μL of potassium iodide at a concentration equal to 2 μg/μL.

RESULTS AND DISCUSSION

HDC Using C1 Column Packed with Porous Particles

Stegeman et al.[17] and Yau et al.[19] have shown that the HDC separation of macromolecules can be attained using porous particles. In this case, when the

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molecular mass increases, the polymers are firstly separated by an SEC mechanism, followed by an HDC mechanism when the molecules are totally excluded from the stationary phase pores. In our study, the C1 column is packed with silica particles having an average pore diameter equal to 100Å.

It is well known, that the protein exclusion limit for such a porous silica-based column is around 150 kDa (20), largely lower than 250 kDa corresponding to the molecular mass of catalase. In the same manner, it has been demonstrated that the small pore size of 100Å hinders the permeation of the pores by DNA fragments larger than 100bp.[9,21] Thus, all the species injected in our chromatographic system were predicted to be excluded from the pores.

As well, Hirabayashi and Kasai[22] have previously reported that columns packed with porous particles developed for the reversed-phase chromatography (such as the C1 column used in this study) can be very useful for the study of the retention of the polymers excluded from the stationary phase pores. In order to eliminate a possible hydrophobic interaction with the particle surface, which could interfere with the hydrodynamic principle, they have used an aqueous mobile phase containing various proportions of acetonitrile.

In our study, when a proportion of the organic modifier was added to the eluent (higher than 10%), no change in the retention time was observed for the different species (data not shown). This demonstrated that the hydrophobic interaction did not exist with the use of 20% of acetonitrile in the eluent.

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Flow Rate Dependence on Retention and Separation in HDC

Using the retention times t_R of the various protein and DNA species and the void time t_0 defined by potassium iodide, an apparent τ value τ_{app} equal to t_R/t_0 was calculated. All the experiments were repeated three times. The variation coefficients of the τ_{app} values were less than 1% in most cases, indicating high reproducibility and good stability for the chromatographic system.

Firstly, the τ_{app} values were plotted against the molecular weight of proteins and plasmids at a flow rate value equal to 0.08 mL/min (Fig. 1). For a similar molecular weight, it was observed that the protein relative retention time was higher than the plasmid τ_{app} value. Similar observations have been made by Boyes et al.[23] Using a SEC GF 250 column, a significant difference in void volumes has been obtained between various excluded proteins and a 2.9 kbp DNA fragment.[23] However, no explanation has been given by the authors. Such phenomenon can be easily explained by the HDC mechanism. It is well established that the globular proteins (as used in this study) are more compact than that of the DNA plasmids.[20] Thus, for the same molecular weight, the hydrodynamic radius of the protein is lower than that of the corresponding plasmid. Following the

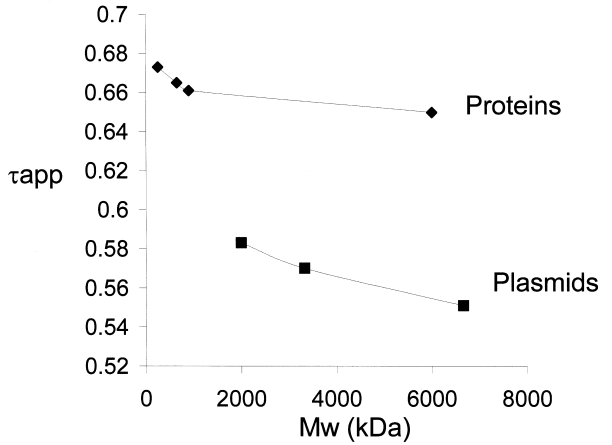

Figure 1. τ_{app} values plotted against the molecular mass of proteins and plasmids at a flow rate value equal to 0.08 mL/min.

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classical HDC theory, the ratio λ increases with the DNA species implying a decrease in the relative retention time.

Secondly, the τ_{app} values were plotted against the mobile phase flow rate for the proteins (Fig 2) and the plasmids (Fig 3). A great difference was observed in the behavior of the two species. The protein curve showed a minimum around

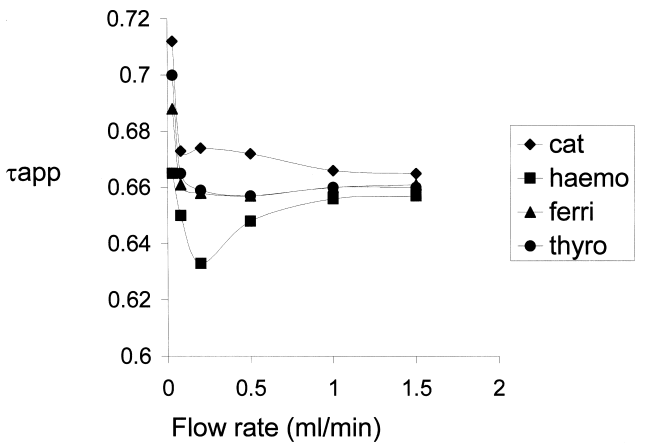

Figure 2. τ_{app} values plotted against the mobile phase flow rate for the proteins.

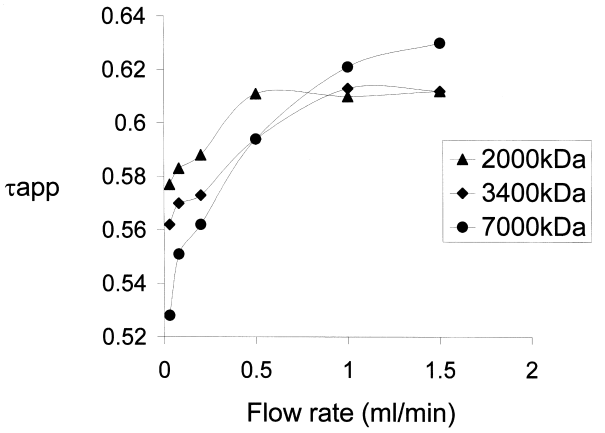

Figure 3. τ_{app} values plotted against the mobile phase flow rate for the plasmids.

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0.02 mL/min, while the plasmid curve increased over the flow rate range. The increase in the plasmid retention with the flow rate was explained by the shear deformation induced by the fluid velocity. Similar observations have been reported by Venema et al.[16] for the behavior of large polystyrenes when the flow rate increases. The authors have concluded that large macromolecules migrate through silica particles "*more or less as a sausage.*"[16] The decline in transverse polymer size was responsible for a decrease in the λ value and then, the retention time increased. It was interesting to note, that this effect was enhanced when the plasmid size increased. This was attributed to the fact that the deformation was facilitated for the high molecular weight as predicted by the Deborah number.

For the protein retention, two phenomena can be distinguished in relation to the flow rate. Below 0.2 mL/min, the relative retention time decreased when the flow rate increased for all the biopolymers. This effect was expected to be attributed to the effect named stress-induced diffusion (SID). SID is due to an entropy gradient in the inter particle capillaries. In a laminar shear flow, the region near the center of the capillary are weakly stressed, while the regions near the walls are highly stressed.[14] The polymers near the walls have a lower entropy than macromolecules in the center of the capillary. This effect is enhanced when the fluid velocity increases. Thus, the polymers, such as proteins, preferred to migrate in the center of the capillary when the flow rate was enhanced and the τ_{app} values decreased (Fig 3). Above 0.2 mL/min, the relative retention increased only for the high size protein.

As described for the plasmids, the high size proteins were submitted to a shear deformation, which was more pronounced for the largest protein, i. e. key-hole lumpet haemocyanin. This implied an increase in the τ_{app}. This deformation was weak, or nil, for the other proteins (due to their lower molecular masses)

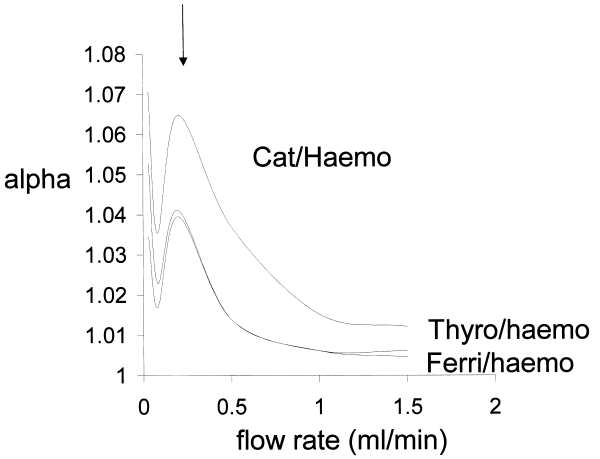

Figure 4. Apparent selectivity α defined as the ratio τ_{app2}/τ_{app1} (1 for keyhole lumpet haemocyanin and 2 for the other proteins) plotted against the flow rate.

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explaining the presence of a plateau. These observations demonstrated that the flow rate effect in HDC balances between two antagonist phenomena, i. e. SID and shear deformation. When the molecule is very compact such as protein, the SID effect plays a great role in the retention dependent on the fluid velocity, while the deformation is only slightly evident at high flow rates. On the other hand, the flexible DNA macromolecules are able to be elongated under the low flow rate[9,13] and then, SID effect is not perceptible.

On the basis of these results, it was possible to obtain the optimal conditions for the HDC separation of proteins. The apparent selectivity, α, defined as the ratio τ_{app2}/τ_{app1} (1 for keyhole lumpet haemocyanin and 2 for the other proteins) was plotted against the flow rate in Fig 4. Two maximal values were observed at the lowest flow rate and around 0.2 mL/min. As the efficiency in packed HDC is roughly independent on the fluid velocity,[17] it can be concluded that the best separation (high α value associated to a low analysis time) is attained at a flow rate equal to 0.2 mL/min.

CONCLUSION

In this paper, it was demonstrated that the separation mechanism of biopolymer in HDC was strongly dependent on the flow rate. The compactness of the macromolecules determined the relative contribution of the two antagonist effects generated by the fluid velocity. When the polymer extension was preponderant (for the random coil DNA plasmid), the retention increased with the flow

rate. On the other hand, this effect was weak, or nil, for globular polymers so that the retention was strongly dependent on entropic effects. From these results, the optimal conditions for the HDC protein separation were attained.

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Received October 24, 2000 Manuscript 5423
Accepted November 10, 2000

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