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Note

Molecular sieving during reversed-phase high-performance liquid chromatography of proteins

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Reversed-phase high-performance liquid chromatography (HPLC) for the separation and isolation of peptides and proteins has become increasingly popular. Only a few systems have been described for the chromatography of large peptides and proteins[1–3]. One of the main problems arises from the general fact that an increase in molecular size results in a concomitant increase in hydrophobicity. This requires relatively high concentrations of organic solvent which may precipitate the proteins[3].

Recently we developed a system for reversed-phase HPLC on a C_{18} support which requires relatively low concentrations of organic solvents[4]. Basically chromatography on a reversed-phase support is dependent on hydrophobicity. Little is known about other factors which affect the separation. Here we present the effect of column size on the retention times of twelve standard proteins.

EXPERIMENTAL

Reagents and materials

Bovine pancreatic ribonuclease, bovine serum albumin, carbonic anhydrase from bovine erythrocytes and whale muscle myoglobin were products from Sigma (St. Louis, MO, U.S.A.). Cytochrome *c* (horse), lysozyme (chicken), *β*-galactosidase (*E. coli*), aldolase (rabbit) and phosphorylase *b* (rabbit) were obtained from Boehringer (Mannheim, G.F.R.). Ferritin (horse) was purchased from Serva (Heidelberg, G.F.R.), ovalbumin from Millipore (Freehold, NJ, U.S.A.) and hemoglobin (horse) was from Pentex Biochemicals (Kankakee, IL, U.S.A.).

Analytical-reagent grade absolute ethanol, synthetic grade ethylene glycol monomethylether (2-methoxyethanol) and sequanal grade trifluoroacetic acid were obtained from E. Merck (Darmstadt, G.F.R.). *n*-Butanol ("Baker" grade) was obtained from Baker Chemicals (Deventer, The Netherlands).

Apparatus

Chromatography was performed on a system consisting of a Waters M 6000 A pump, an LKB 11300 Ultrograd gradient mixer, a Rheodyne 7125 injector and a Pye Unicam LC-UV detector operated at 215 nm. Columns (30 × 3, 180 × 3 and 300 × 4.6 mm) were slurry-packed with Nucleosil 10 C_{18} (Macherey, Nagel & Co., Düren, G.F.R.).

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Chromatography

The proteins were chromatographed at a flow-rate of 1 ml/min with a gradient made of solvents A and B. Solvent A consisted of water–2-methoxyethanol–*n*-butanol–trifluoroacetic acid (87:10:3:0.1), solvent B was ethanol–*n*-butanol–2-methoxyethanol–trifluoroacetic acid (70:20:10:0.025) (all v/v). The rate of solvent change was 2.5%/min.

RESULTS AND DISCUSSION

The retention times of twelve standard proteins on three different columns with volumes of 0.21, 1.27 and 4.98 ml, respectively, are given in Table I. An example of the separation on the short column (30 × 3 mm) is shown in Fig. 1. From Table I it can be seen that the relative retention times are dependent on the size of the column used. For some proteins this results in a reversal of the elution order, for example for myoglobin and aldolase. With other proteins, baseline separations are obtained on one column, whereas they co-elute on the other column, as is the case with bovine serum albumin and lysozyme (see Fig. 1 and Table I). Intermediate values are obtained for the proteins chromatographed on the medium-size column. With decreasing molecular weight, proteins are relatively more retarded on the longer columns.

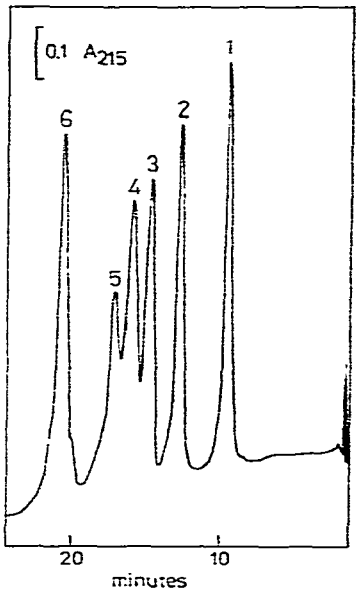
TABLE I

RETENTION TIMES (min) OF PROTEIN STANDARDS

Proteins (20–30 μg) were chromatographed on columns I (30 × 3 mm), II (180 × 3 mm) and III (300 × 4.6 mm), as described in the Experimental section.

Protein	*Column*		
	I	*II*	*III*
Ribonuclease	11.3	13.3	16.5
Cytochrome *c*	14.8	16.5	18.9
Lysozyme	15.0	17.0	19.6
Bovine serum albumin	16.9	18.3	19.9
β-Galactosidase	17.0	18.3	19.9
Myoglobin	18.2	20.0	22.0
Aldolase	18.7	20.1	21.8
Carbonic anhydrase	18.8	20.6	22.6
Haemoglobin	19.6	21.6	23.8
Ferritin	21.9	23.8	25.8
Phosphorylase *b*	22.3	23.4	25.0
Ovalbumin	23.0	24.6	26.4

The difference between the retention times after chromatography of the standard proteins on both the long and the small column was plotted against the molecular weight (Fig. 2). Among the proteins studied were multimeric proteins of which aldolase, ferritin and haemoglobin could be easily fitted to the curve in Fig. 2 if it is assumed that these proteins are in their dissociated monomeric form. Such a dissociation could be caused by the composition of the eluent and/or the high pressure. This cannot be deduced for phosphorylase *b* and β-galactosidase since both the dissociated



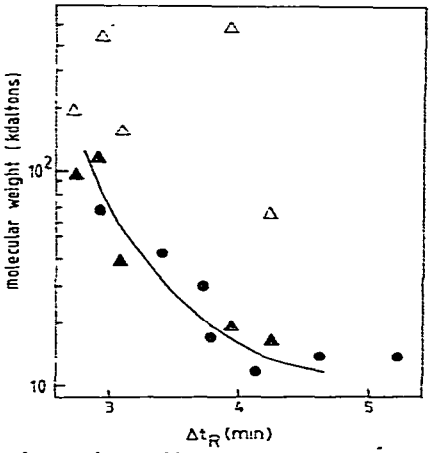


Fig. 1. Chromatography of protein standards on a C_{18} reversed-phase column (30 × 3 mm). Proteins (20–30 μg) were chromatographed at a flow-rate of 1 ml/min as described in the Experimental section, except that in this case the gradient consisted of two linear segments. (i) from 100% solvent A to 10% B in A in 1.5 min and (ii) to 60% B in A in 21 min. Peaks: 1 = ribonuclease; 2 = lysozyme; 3 = bovine serum albumin; 4 = myoglobin; 5 = haemoglobin; 6 = ovalbumin.

Fig. 2. The difference in retention time (Δt_R) between chromatography on the long column (300 × 4.6 mm) and on the short column (30 × 3.0 mm) against the molecular weight of the protein standards. ● = proteins consisting of one single polypeptide chain; △ = proteins consisting of more than one subunit; ▲ = monomeric form of the multimeric proteins.

and undissociated forms of these proteins do fit the curve in Fig. 2. Despite the latter uncertainty these results suggest a relationship between the molecular weight and the relative retention time of each protein chromatographed on columns of different size. This apparent molecular-sieving effect could be the result of the small pore size (100 Å) of the octadecyl-coated silica particles.

Although the hydrophobicity of peptides and proteins increases generally with molecular weight[3] the elution order of proteins does not correlate with molecular weight[5]. This study, however, shows that the molecular weight still has a minor but important impact on the retention time. It emphasizes the importance of column size and pore size for reversed-phase HPLC of proteins.

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