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Note

Rapid analysis of proteins and peptides by reversed-phase chromatography

KRISHNA KALGHATGI and CSABA HORVÁTH*
Department of Chemical Engineering, Yale University, P.O. Box 2159 Yale Station, New Haven, CT 06520 (U.S.A.)
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Modern liquid chromatography commenced with the introduction of pellicular sorbents that were stable at high pressures and temperatures and yielded columns of high efficiency at flow velocities much higher than those used in traditional column chromatography[1,2]. The advent of microparticulate bonded phases which afforded columns of even higher efficiencies and loading capacities virtually eliminated the need for traditional pellicular sorbents made of relatively large-size glass beads as the support. Only recently have micropellicular sorbents of 1.5 or 7 μm particle diameter been prepared for protein separations by reversed-phase[3] and ion-exchange[4] chromatography. The significance of using such sorbents rests with the gain in column efficiency that is higher with pellicular than with totally porous particles particularly at relatively high flow velocities[5] encountered with large molecules such as proteins by virtue of their low diffusivity.

EXPERIMENTAL

Materials

Ribonuclease A, cytochrome *c*, lysozyme, β-lactoglobulin A, L-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, U.S.A.). L-Aparaginase was from Merck, Sharp and Dohme (West Point, PA, U.S.A.) and methionyl human growth hormone was a gift from Genentech (San Francisco, CA, U.S.A.). HPLC-grade acetonitrile and reagent-grade phosphoric acid were obtained from Fisher Scientific (Springfield, NJ, U.S.A.). Mobile phases were prepared with NanoPure (Barnstead) deionized water.

Silica microspheres (2 μm) were prepared by controlled hydrolysis of tetraalkylsilicate[6] and the surface of the monodisperse, non-porous support was treated with *n*-octyldimethylchlorosilane[7].

HPLC equipment

The instrument embodied a Series 400 pump, and a Model LC-95 UV–VIS detector from Perkin-Elmer. Chromatograms were obtained with a Shimadzu C-R3A Chromatopac integrator. The eluent from the pump passed through a heat exchanger

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coil, a Rheodyne Model 7025 injection valve and the column; all of them were kept in a constant-temperature bath (Haake) filled with ethylene glycol. The dead volume of fittings and tubings was kept at a minimum and the flow cell of the detector was pressurized. The eluent reservoirs were purged with helium. The 10 × 4.75 mm column was made from a Parker Hannifin union (No. 4-4 HBZ-SS-C), whereas the 30 × 4.6 mm column had the same construction as conventional high-performance liquid chromatography (HPLC) columns. Both were packed using a methanol slurry of the sorbent at 70 MPa. All plumbings, fittings and 0.5-μm frits were No. 316 stainless steel.

Tryptic digestion

Proteins (2 mg ml^{-1}) were digested at 37°C with TPCK-treated trypsin at an enzyme to substrate ratio 1:100 in solutions containing 100 m*M* sodium acetate, 10 m*M* Tris and 0.1 m*M* calcium chloride, pH 8.3. The reaction was stopped after 4 h by adding 10%, phosphoric acid and the protein digests were stored at 4°C.

RESULTS AND DISCUSSION

Rapid analysis

The main objective of this investigation was to demonstrate high-speed analysis of proteins and peptides at high flow-rates and elevated temperature by using short columns packed with a non-polar micropellicular sorbent. As illustrated in Fig. 1 a mixture of five proteins was separated in about 20 s and chromatograms of tryptic digests of β-lactoglobulin A and methionyl human growth hormone shown in Fig. 2 were obtained in 1 and 3 min, respectively. The viscosity of the hydro-organic phase was reduced by increasing the temperature so that the column could be operated at

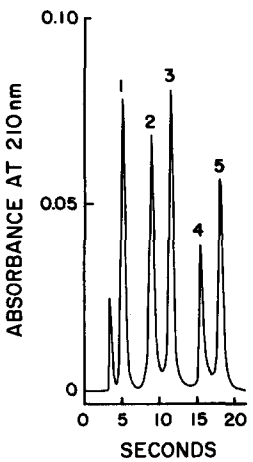

Fig. 1. Chromatogram of a protein mixture. Column: 30 × 4.6 mm, packed with 2-μm fluid-impervious spherical silica particles having covalently bound *n*-octyl functions at the surface. Linear gradient in 48 s from 15 to 95% acetonitrile in water containing 0.1% TFA. Flow-rate: 4 ml min^{-1}. Temperature: 80°C. Column inlet pressure: 30 MPa. Sample components: 1 = ribonuclease A, 50 ng; 2 = cytochrome *c*, 25 ng; 3 = lysozyme, 25 ng; 4 = L-asparaginase, 50 ng; 5 = β-lactoglobulin A, 25 ng. Detection at 210 nm.

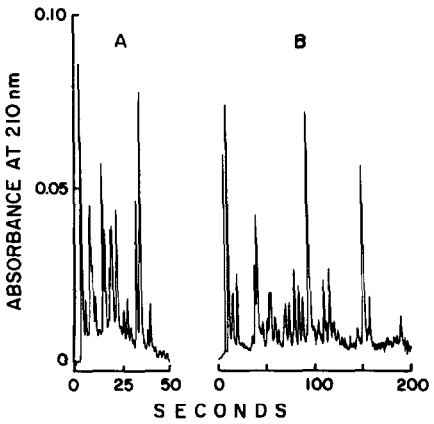

Fig. 2. Chromatographic profiles of tryptic digests of (A) β-lactoglobulin A and (B) methionyl human growth hormone. Conditions as in Fig. 1. except the linear gradients from 0 to 95% acetonitrile in water containing 0.1% TFA (A) in 2 min at 5 ml min^{-1} and (B) in 6 min at 4 ml min^{-1}. Each sample corresponds to 5 μg of protein digest.

relatively high flow velocities without exceeding the pressure limitations of standard HPLC equipment. Due to the favorable mass transfer characteristics of the pellicular sorbent configuration[1], as well as the fast diffusion rates and sorption kinetics at elevated temperature, the efficiency of the column is largely retained at high flow velocities. The key to practicability of this approach is the longevity of the column at temperatures near the atmospheric boiling point of mobile phase.

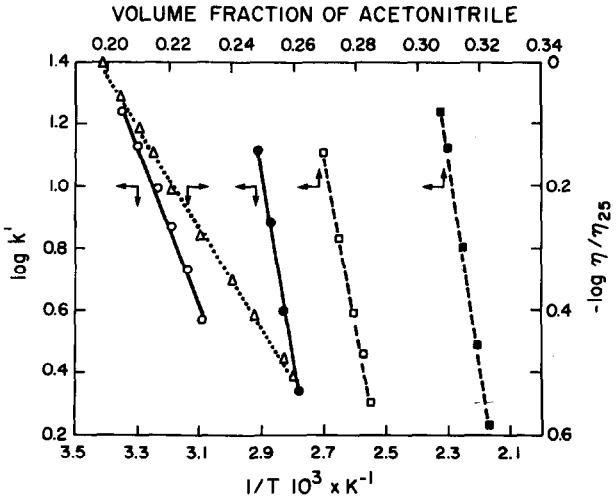

Fig. 3. Plots of the logarithmic retention factor (k') for lysozyme against the reciprocal temperature and isocratic eluent composition. Column as in Fig. 1. Data for Van 't Hoff plots were obtained with 30.75% (○) and 28% (●) acetonitrile in water containing 0.1% TFA. The effect of eluent compositions was investigated at 25°C (■) and 80°C (□). The sample size was 5 ng. The plot of logarithm of eluent viscosity relative to the viscosity at 25°C, η/η_{25}, against the reciprocal temperature is shown by the dotted line. Each data point represents values obtained with eluents of different compositions in the range from 15 to 60% acetonitrile in water containing 0.1% TFA.

Effect of temperature and organic modifier

Andrade plots[8] of the logarithmic viscosity of various water–acetonitrile mixtures against the reciprocal temperature were linear and parallel, with slopes corresponding to an activation energy of 4.0 kJ mol^{-1} as shown in Fig. 3. Thus, the viscosity of the eluent and the column inlet pressure at a fixed flow-rate, are reduced three-fold by increasing the temperature from 20 to 80°C. Viscosity was calculated according to Darcy's law from the column inlet pressure at different eluent composition, temperature and flow-rates by using $1.00 \cdot 10^{-3}$ N s m^{-2} and $0.37 \cdot 10^{-3}$ N s m^{-2} as the respective values for water and acetonitrile at 20°C[9].

The retention of lysozyme was investigated with isocratic elution and Fig. 3 shows plots of the logarithmic retention factor against the solvent composition and the reciprocal temperature. Slopes of both types of plots are much greater than those obtained with small-molecular-weight eluites under similar conditions. The retention factor is particularly sensitive to small changes in the organic solvent content of the eluent; yet, with the 30 × 4.6 mm column and gradient elution (see Figs. 1 and 2), the results were highly reproducible. The retention enthalpies calculated from the slope of the Van 't Hoff plots are 12.5 and 33.5 kJ mol^{-1} at 30.75% and 28% acetonitrile, respectively. These values are about 3 to 8 times higher that those encountered in reversed-phase chromatography of small molecules[7].

Protein separation by isocratic elution

The relatively high efficiency of the system also permits rapid separation of closely related proteins by isocratic elution, which may be of interest in process monitoring. The chromatographic separation of β-lactoglobulins A and B, which differ only in two amino acid residues, is depicted in Fig. 4 to illustrate the potential of the method. With isocratic elution, however, the loading capacity of such a small columns was rather low, retention times and to a lesser extent bandwidths are independent of the sample size only in the submicrogram range and restrict their use to analytical chromatography.

The results demonstrate that the separation of high-molecular-weight sub-

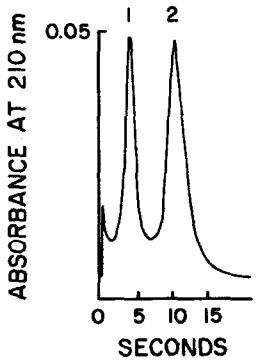

Fig. 4. Separation of β-lactoglobulin B (1) and A (2) by isocratic elution. Column: 10 × 4.6 mm, packed with the micropellicular sorbent described in Fig. 1. Eluent: 36.75% acetonitrile in water containing 0.1% TFA. Flow-rate: 3 ml min^{-1}. Temperature: 80°C. Column inlet pressure: 14.7 MPa. Sample size: 10 ng. Detection at 210 nm.

stances on a time scale of seconds is possible by the use of columns packed with suitable micropellicular stationary phases which are stable at high operating temperatures where the potential of such sorbents can best be exploited. Further advances in instrument design, however, are needed to reduce dead volume and facilitate rapid heating and mixing of the eluents that are requisites for gradient elution and column regeneration on a timescale commensurate with the actual time of separation.

We envision numerous applications of this technique to rapid analysis of biopolymers when the sample components do not have to be recovered in their native forms. Similar results are being obtained with nucleic acids, as well as with proteins, by using micropellicular sorbents with polystyrene-based supports at high temperatures and eluent pH.

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REFERENCES

1 Cs. Horváth, B. A. Preiss and S. R. Lipsky, *Anal. Chem.*, 39 (1967) 1422.
2 J. J. Kirkland, *Anal. Chem.*, 41 (1969) 218.
3 K. K. Unger, G. Jilge, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 359 (1986) 61.
4 D. J. Burke, J. K. Duncan, L. C. Dunn, L. Cummings, C. J. Siebert and G. S. Ott, *J. Chromatogr.*, 353 (1986) 425.
5 Cs. Horváth and S. R. Lipsky, *J. Chromatogr. Sci.*, 7 (1969) 109.
6 W. Stober, A. Fink and E. Bohn, *J. Colloid Interface Sci.*, 26 (1968) 62.
7 W. Melander and Cs. Horváth, in Cs. Horváth (Editor), *High Performance Liquid Chromatography — Advances and Perspectives*, Vol. 2, Academic Press, New York, 1980, p. 114.
8 E. N. C. Andrade, *Nature (London)*, 125 (1930) 582.
9 H. Colin, J. C. Diez-Masa, G. Guiochon, T. Czajokowska and I. Miedziak, *J. Chromatogr.*, 167 (1978) 41.