Journal of Chromatography, 499 (1990) 267–278
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1665

MICROPELLICULAR STATIONARY PHASES FOR RAPID PROTEIN ANALYSIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KRISHNA KALGHATGI
Department of Chemical Engineering, Yale University, New Haven, CT 06520-2159 (U.S.A.)

SUMMARY

The high separating speed, efficiency and operational stability of various micropellicular stationary phases are demonstrated in the high-performance liquid chromatography (HPLC) of biopolymers. The micropellicular sorbents were prepared from 2-μm fluid-impervious silica microspheres as the support, with a thin layer of different retentive materials at the surface. These include a molecular fur of octyl or stearyl chains for reversed-phase chromatography as well as a hydrophilic layer with amino groups and polyethyleneglycol chains for anion-exchange and hydrophobic interaction chromatography, respectively. The use of appropriate micropellicular stationary phases for protein separation by metal-interaction and affinity chromatography is also illustrated. In most cases, operation at elevated column temperature was found to be preferable for rapid separations. Preliminary results show that the stability of micropellicular columns compares very favorably with that of columns conventionally used in HPLC and that they are easy to maintain.

INTRODUCTION

Reversed-phase chromatography (RPC) continues to be a leading analytical technique for separation of proteins and peptides. The success of this method is due to its general applicability, availability of highly efficient columns and of highly sophisticated (HPLC) instrumentation. By virtue of its versatility, RPC has successfully met the new challenges of modern biotechnology in both analytical and preparative chromatography of biological macromolecules[1–3]. Recently, there has been considerable interest in further improvement of speed and column efficiency by employment of micropellicular stationary phases for the separation of biopolymers [4–11]. In comparison to the usual porous sorbents, pellicular sorbents allow faster mass transfer due to the absence of intraparticle diffusional resistances. This leads to higher column efficiency, particularly at relatively high flow velocities and for large molecules with low diffusivity. Due to the solid, fluid-impervious core and low surface area, the micropellicular stationary phases are generally more stable at elevated temperature than conventional, porous column materials. An increase in temperature results in the improvement of sorption kinetics as well as an increase in solute diffusivity, with a concomitant decrease in the viscosity of the mobile phase. Therefore, use of small-

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particle, pellicular stationary phases and elevated temperatures, together, are expected to facilitate rapid analysis of proteins, peptides and other biopolymers.

Historical background

The role and importance of mass transfer in chromatographic processes is well described in the literature[12–15] and therefore, appropriate choice of conditions that provide favorable mass-transfer properties is essential for high-efficiency separations. Particle size is an important parameter contributing to the efficiency of the column and reduction in particle size leads to greater column efficiency. However, this approach is generally restricted by the concomitant decrease in column permeability due to excessive pressure. Another important factor is the support material itself. In column liquid chromatography of proteins the packing material has consisted of porous particles, which are characteristically weak and have a low efficiency, due to diffusional resistances in the stagnant mobile phase in the retentive material. Attempts to improve mass-transfer properties of the stationary phase have been met, with limited success, in the 1950s by the use of surface-coated packings, such as Celite, coated with a layer of ion-exchange resins for the separation of proteins[16,17] or by the use of polyethylenimine (PEI)-coated cellulose for the separation of nucleic acid constituents by thin-layer chromatography[18]. With the advent of HPLC, another approach was used to reduce diffusional resistance to mass transfer by reducing the diffusional path length in the stationary-phase support. Horváth and co-workers[19–24] were first to demonstrate the merits of pellicular stationary phases in HPLC, and this approach was further pursued by Kirkland[25,26]. The separation of nucleic acid constituents on the pellicular ion exchangers, prepared from relatively large glass beads [particle size (d_p) $\approx$ 40 μm] as supports, marked an advance in stability at elevated temperature and pressure, in separation efficiency, and in high-speed analysis by HPLC. The concept of pellicular sorbents was successfully extended also to the use of immobilized enzymes[27,28]. In the early years of HPLC, a wide variety of these sorbents found commercial applications[15]. They were made from relatively large glass beads and used for the separation of small molecules.

Advances in particle classification technology in the late sixties led to the commercial availability of 5- or 10-μm particles with narrow particle size distribution. This was the beginning of an era of bonded phases on totally porous microparticulate supports. This brought about the decline in the use of pellicular sorbents of much greater particle size. The microparticulate porous bonded phases had greater efficiency and higher sample load capacity. In addition, availability in the last decade of a wide variety of totally porous supports in a wide range of particle sizes and porosities has established such porous microparticulate stationary phases as a standard in HPLC of small molecules.

In recent years HPLC has made very significant advances in the separation of large molecules of biological origin. Such compounds are generally analyzed by gradient elution by the use of columns packed with small particles (3–10 μm in size) with relatively large pores (300–500 Å). However, low diffusivity of biopolymers and restricted mass transfer in the porous interior of the column packings often results in long analysis times for high-molecular-weight substances. Even the macroporous (pore size $>$ 50 nm) stationary phases have been described as showing poor performance, as well as low recovery of mass and biological activity[5]. Although an increasing

pore diameter is expected to alleviate some of these problems, particles with very large pores (> 1000 Å) do not possess sufficient mechanical strength for use in HPLC. In the mid-1980s, Unger and co-workers[5,29,30] revived the concept of pellicular sorbents, and introduced 1.5-μm, monodisperse, non-porous, reversed-phase silica packings into biopolymer chromatography. This was followed by other micropelllicular sorbents, based on siliceous[8–10,31], or polymeric[4,7,11,32,33] supports for the HPLC of large molecules. Recent work[8–10,33] from our laboratory has demonstrated the merits of micropellicular sorbents in allowing high speed of analysis and high column efficiency. Furthermore, their superior stability at elevated temperature for protein separation and peptide mapping, has also been established[8]. This study illustrates the versatility of micropellicular stationary phases in HPLC for rapid separation of proteins by different types of chromatography.

EXPERIMENTAL

Materials

Insulin, ribonuclease A, α-chymotrypsinogen A (all from bovine pancreas), cytochrome *c* (horse heart), lysozyme (chicken egg white), myoglobin (sperm whale), β-lactoglobulin A (bovine milk), ovalbumin (chicken egg), concanavalin A (Con A) (jack bean), α-methylmannoside and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, U.S.A.). r-Human growth hormone (r-hGH) and tissue plasminogen activator (r-tPA) were from Genentech (South San Francisco, CA, U.S.A.). N-Tosyl-L-phenylalanine chloromethylketone (TPCK)-treated trypsin was obtained from Worthington (Freehold, NJ, U.S.A.). Iminodiacetic acid (IDA) disodium salt, ethylene diamine tetraacetic acid (EDTA) tetrasodium salt, polyethylene glycol (PEG) 600, polyethylenimine (PEI) 600, γ-glycidoxypropyltrimethoxysilane, boron trifluoride etherate, $NiSO_4 \cdot 7H_2O$ were obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC-grade acetonitrile, methanol, reagent-grade orthophosphoric acid and buffer salts were from J. T. Baker (Phillipsburg, NJ, U.S.A.). Tetraethylammonium hydroxide (TEAH) and octylsodium sulfate were products of Eastman Kodak (Rochester, NY, U.S.A.). Eluents were prepared with deionized water, prepared with a NanoPure system (Barnstead, Boston, MA, U.S.A.), filtered through a 0.45-μm filter, and degassed by sparging with helium before use.

Instruments

A Hewlett Packard Model 1090 Series M liquid chromatograph (Avondale, PA, U.S.A.), equipped with a ternary DR5 solvent delivery system, diode-array detector, ColorPro graphic plotter and autosampler, were used. The chromatographic system and data evaluation were controlled by Series 79994A Chem Station computer. The column effluent passed through heat exchangers in the diode-array detector before entering the flow cell.

Other experiments wre carried out with a Series 400 pump and a Model LC 95 detector, both from Perkin Elmer (Norwalk, CT, U.S.A.), which were assembled with a heat exchanger coil and a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.). temperature circulating bath (Haake Buchler, Saddlebrook, NJ, U.S.A.). The flow cell of the detector was pressurized, and the chromatograms were processed by C-R3A Chromatopak integrator (Shimadzu, Columbia, MD, U.S.A.).

Column stability measurements were performed by using a chromatograph assembled together with a Series 10 pump, Model LC 65T detector/oven (both from Perkin Elmer), and a Model 728 autosampler from (Micromeritics, Norcross, GA, U.S.A.). Silica microspheres were sized by Model CAPA-700 particle size analyzer (courtesy of M. Perlstein, Horibo Instruments, Irvine, CA, U.S.A.).

Columns

Non-porous silica microspheres (d_p = 2 μm) and Hy-Tach C_{18} micropellicular reversed-phase columns were obtained from Glycotech (Hamden, CT, U.S.A.). Concanavalin A (Con A) was bound to non-porous silica microspheres according to the procedure described previously[10]. The micropellicular anion exchanger was prepared by reaction with γ-glycidoxypropyltrimethoxysilane to form epoxy silica, followed by treatment with PEI, according the procedure of Regnier and Noel[34]. Similarly, the micropellicular stationary phase for hydrophobic interaction chromatography (HIC) was prepared by reaction of epoxy silica with PEG in the presence of boron trifluoride. In most cases, the stationary phases were packed into 30 × 4.6 mm I.D. columns. The 0.5-μm dual-density frits (Mott Metallurgical, Farmington, CT, U.S.A.) and connecting lines were made of No. 316 stainless-steel.

Experiments with a totally porous stationary phase were carried out by using a Vydac C_{18} column (Type 218TP54, 250 × 4.6 mm I.D., Separations Group, Hesperia, CA, U.S.A.).

Procedures

Reduction, S-carboxymethylation of r-tPA, and subsequent digestion with trypsin were carried out according to the procedure described previously[9]. Sample injections were made to coincide with the commencement of the gradient, and actual gradient profiles were determined by tracer technique[9].

RESULTS AND DISCUSSION

Stability of micropellicular sorbents

A scanning electron micrograph of non-porous silica microspheres is shown in Fig. 1. These particles have a nominal diameter of 2 μm and exhibit a narrow particle size distribution, as shown in Fig. 2. The specific surface area was found to be 1.20 m^2/g by the BET method. This is in agreement with the calculated value (1.35 m^2/g) for the geometric surface area, of solid spheres 2 μm in diameter and, thus, suggests the absence of internal pore structure in the silica microspheres. Due to very small size and lack of porosity, the columns packed with such silica particles are expected to be much more stable at high inlet pressures and elevated temperature than traditional HPLC packings made from porous silica or polymeric supports. The stability of micropellicular C_{18} stationary phase was investigated at elevated temperature in acidic and moderately alkaline medium. The column performance was tested for the separation of standard proteins at various stages of elution with the mobile phases given in Table I. The chromatograms in Fig. 3 showed no sign of deterioration of the column after pumping 35 000 and 30 500 column void volumes of acidic and alkaline eluents, respectively, at 80°C. Stability was also tested under conditions analogous to those employed in protein chromatography for cleaning of the column by injecting an

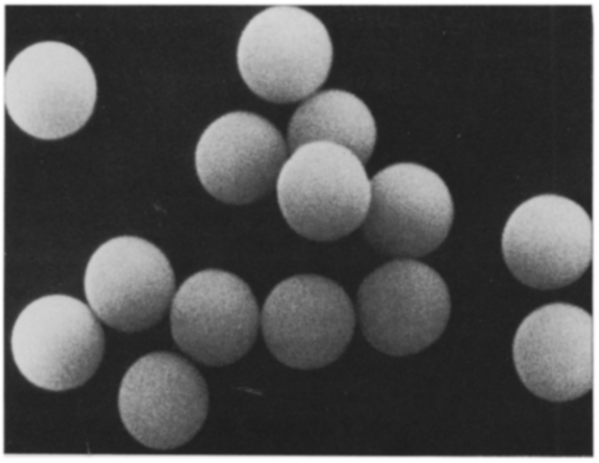


Fig. 1. Electron micrograph of micropellicular silica.

appropriate cleansing agent. Micropellicular C_{18} sorbents were stable after 128 injections (70 μl each) of 0.25% TEAH (pH 12), and perfusion with 6000 column void volumes of the mobile phase at pH 9.0. However, the column performance was severely affected by injection of TEAH at a higher concentration (1%, pH 13) or 0.1 *M* sodium hydroxide.

Reversed-phase chromatography

Rapid analysis. RPC is an increasingly used method of analysis for proteins and peptides. It is employed successfully for the determination of sample composition, assay of purity for the detection of trace impurities in protein samples, and for monitoring of biological processes[1,2]. Previous work from this laboratory has shown that silica-based micropellicular stationary phases can be successfully used for rapid protein analysis[8], peptide mapping[9] and trace analysis[35]. The absence of pore structure in micropellicular sorbents, which provide significant advantages, such as complete

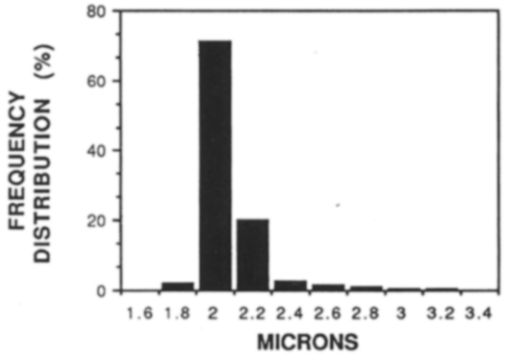


Fig. 2. Particle size distribution of micropellicular silica. A suspension of sillica beads (100 mg) in 25 ml of water was sonicated for 30 min and the particle size was analyzed with a Model CAPA-700 particle size analyzer.

TABLE I

STABILITY OF MICROPELLICULAR STATIONARY PHASES

Micropellicular C_{18} columns, 30 × 4.6 mm I.D., were maintained at 80°C in an oven and perfused with the eluents listed below. The performance of the column at each step was tested for separation of proteins, as described in Fig. 3.

Column perfusate	*pH*	*Volume*[a]	*Injection*[b]	*Test of efficiency*[c]
Methanol–water (1:1, v/v) + 0.1% TFA	2.0	3500	–	NC
Methanol–water (45:55, v/v) + 0.1% TFA	2.0	8000	–	NC
Methanol–water (15:85, v/v) + 0.1% TFA	2.0	5500	–	NC
Methanol–water (15:85, v/v) + 0.1% TFA	2.0	9000	–	NC
50 m*M* Ammonium bicarbonate	8.5	9000	–	NC
50 m*M* Tris–orthophosphoric acid	8.5	9000	–	NC
Methanol–50 m*M* Tris (15:85, v/v)	9.0	12500	–	NC
50 m*M* Trisodium phosphate	11.0	5500	–	LE
50 m*M* Tris–phosphoric acid	8.5	6000	0.25% TEAH	NC
50 m*M* Tris–phosphoric acid	8.5	3000	1.0% TEAH	LE
50 m*M* Tris–phosphoric acid	8.5	3000	0.1 *M* NaOH	LE

[a] Expressed as column void volumes.
[b] Sample (70 μl) injected 64 times at 5-min intervals during perfusion of the column.
[c] NC = no change in column performance; LE = loss of column efficiency.

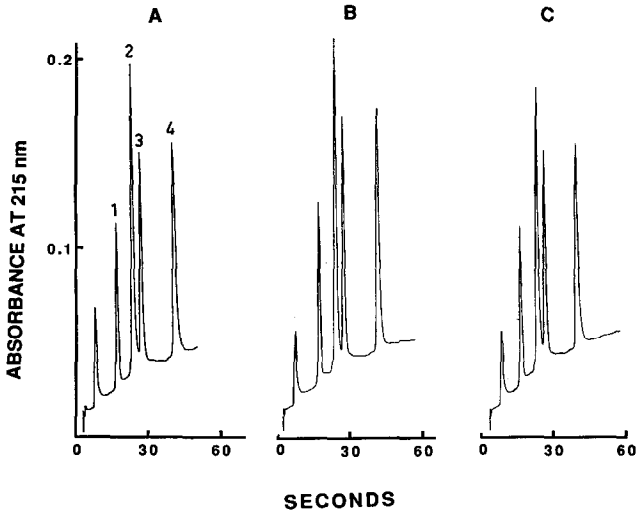
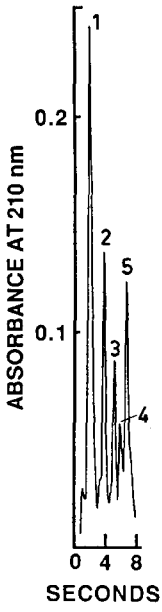


Fig. 3. Stability of micropellicular C_{18} stationary phase. Columns (30 × 4.6 mm I.D.) were eluted with various mobile phases under conditions described in Table I. Sample, 20 μl, containing 0.5 μg each of ribonuclease A (1), cytochrome *c* (2), lysozyme (3) and β-lactoglobulin A (4); flow-rate, 3 ml/min; temperature, 80°C; eluent A, 0.1 (v/v) aqueous TFA; eluent B, 95% aqueous acetonitrile containing 0.1% (v/v) TFA. The sample components were eluted by a linear increase of eluent B from 20 to 90% in 1.5 min. Chromatogram A was obtained at initial stage of a newly packed column, whereas B and C were obtained with the same column after elution with 26 000 column void volumes of aqueous methanol containing 0.1% (v/v) TFA, 9000 column void volumes each of 50 m*M* NH_4HCO_3, Tris–orthophosphoric acid (both pH 8.5) and 50 m*M* Tris (pH 9.0).

exposure to large molecules and rapid solute equilibration with the stationary phase, is demonstrated in Fig. 4 by high-speed analysis of five proteins in less than 8 s. Current practice of protein chromatography does not require analyses to be carried out in seconds, and analysis on a time scale of seconds imposes additional constraints on the instrumentation[35–37]. The results presented here demonstrate the potential of micropellicular sorbents for fast analysis of biological macromolecules.

Comparison with totally porous stationary phases. Columns packed with micropellicular (2 μm) and totaly porous (5 μm; 300 Å) reversed-phase sorbents were used for the separation of proteins. The analyses were carried out at constant temperature. Column inlet pressure and gradient conditions were optimized for each column to achieve base-line resolution of all components. Although both columns provided excellent resolution of five proteins at room temperature, the analysis time was three times shorter with the micropellicular column (Fig. 5A and B). Since the speed of analysis is proportional to the velocity of the mobile phase, it is necessary to operate the column at flow-rate as high as possible without significant loss in separation efficiency. This approach is limited by (i) pressure constraints due to low permeability of the columns packed with micropellicular sorbents and (ii) significant departure from the minimum of the Van Deemter curve, resulting in loss of efficiency for porous particles[15]. At elevated temperature, the solute diffusivity and sorption rates increase,



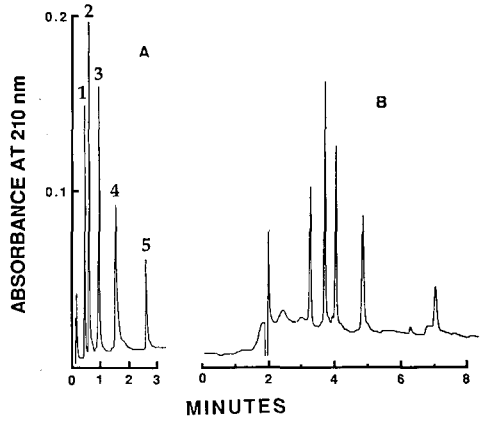


Fig. 4. High-speed analysis of proteins. Column, 5.0 × 4.6 mm I.D. micropellicular C_{18}; flow-rate, 5 ml/min; temperature, 80°C; sample, *ca.* 100 ng each of ribonuclease A (1), cytochrome *c* (2), lysozyme (3), L-asparaginase (4) and β-lactoglobulin A (5). Elution was carried out by a linear gradient of acetonitrile from 18 to 90% in 30 s.

Fig. 5. Reversed-phase chromatography of proteins on micropellicular (A) and totally porous (B) stationary phases. Sample components were ribonuclease A (1), insulin (2), lysozyme (3), myoglobin (4) and r-hGH (5); temperature, 22°C. For details see Table II.

TABLE II

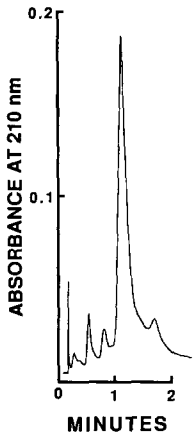
COMPARISON OF OPERATING CONDITIONS IN THE REVERSED-PHASE CHROMATOGRAPHY OF PROTEINS BY MICROPELLICULAR AND TOTALLY POROUS STATIONARY PHASES

Column	*Temperature (°C)*	*Flow-rate (ml/min)*	*Pressure (p.s.i.)*	*Gradient*	*Analysis time (min)*	*Regeneration time[a] (min)*	*Cycle time (min)*	*Solvent consumption per run (ml)*
Micropellicular	22	1.3	2530	15–90% B in 4 min	2.5	1.5	4.0	5.2
(30 × 4.6 mm I.D.)	40	1.7	2600	15 90% B in 1.5 min	1.1	0.9	2.0	3.4
Total porous	22	1.3	1800	15–90% B in 9 min	7.8	7.0	14.8	19.2
(250 × 4.6 mm I.D.)	40	2.0	1800	15–90% B in 9 min	7.3	6.0	13.3	26.6

[a] Determined by the time required for the detector signal to return to initial value.

leading to reduction of plate height and abatement of pressure restrictions[38]. In contrast with porous supports, the micropellicular packings can be used at elevated temperatures without adverse effect on column performance. Due to the absence of pores in micropellicular sorbents, columns packed with such stationary phases can be regenerated faster than those with porous stationary phases after gradient elution. A comparison of operating conditions and productivity of protein chromatography with micropellicular and totally porous stationary phases is given in Table II. Whereas both types of columns yielded satisfactory results for the resolution of sample components, the micropellicular column had a shorter cycle time and consumed less solvent than the column with porous stationary phase. On the other hand, the columns with conventional porous stationary phases have certain advantages, such as greater column permeability and higher load capacity, which can be important in certain applications.

Other methods of protein chromatography. The surface of non-porous silica microspheres was modified with appropriate functions and the stationary phases thus obtained were used in the separation of proteins by ion-exchange HIC and metal-interaction chromatography (MIC). A commercial sample of ovalbumin was analyzed in 2 min with a salt gradient on a micropellicular PEI column. Various peaks in Fig. 6 represent the microheterogeneity of ovalbumin due to glycosylation and phosphorylation of the protein[39,40]. Figs. 7 and 8 illustrate the rapid separation of proteins by HIC and MIC, respectively.



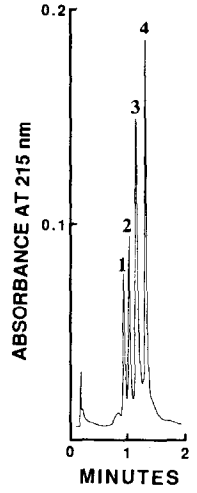
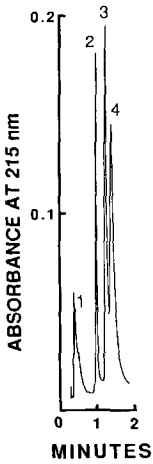


Fig. 6. Ion-exchange chromatography of commercial ovalbumin. Column, micropellicular PEI (30 × 4.6 mm I.D.). The sample components were eluted by a linear increase of NaCl from 0 to 0.5 *M* in 5 min with 25 m*M* phosphate buffer (pH 7.5). The main peak represents ovalbumin and its glycosylated/phosphorylated conformers are shown by the minor peaks. Flow-rate, 2 ml/min; temperature, 70°C.

Fig. 7. Hydrophobic interaction chromatography. Column: micropellicular polyether (30 × 4.6 mm I.D.); flow-rate, 3 ml/min; temperature, 50°C. Sample components, cytochrome *c* (1), ribonuclease A (2), lysozyme (3) and α-chymotrypsinogen (4). Eluent, decreasing gradient of ammonium sulfate from 3 to 0 *M* in 3 min.



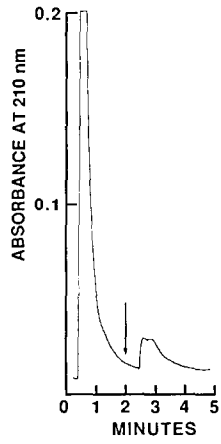


Fig. 8. Metal-interaction chromatography. Column, micropellicular IDA complexed with nickel (30 × 4.6 mm I.D.). Sample components: β-lactoglobulin A (1), chymotrypsinogen A (2), cytochrome *c* (3) and lysozyme (4). Eluent, linear gradient of NaCl from 0 to 0.5 *M* in 3 min; flow-rate, 2 ml/min; temperature, 50°C.

Fig. 9. Separation of glycopeptides and non-glycopeptides in a tryptic digest of r-tPA. Column, micropellicular Con A (30 × 4.6 mm I.D.). The protein digest (25 μg) was injected and the bound fraction, containing the glycopeptides, was eluted with 50 m*M* α-methylmannoside (2 min) in the starting eluent, consisting of 25 m*M* Tris–0.15 m*M* NaCl (pH 7.5); flow-rate, 1 ml/min; temperature, 25°C.

Combination of chromatographic techniques (LC–LC). Since micropellicular stationary phases are suitable for rapid analysis of proteins and peptides on a time scale of a few minutes or less, it seemed appropriate to explore a combination of more than one chromatographic technique (LC–LC) for the separation of complex mixtures of biological substances. An example shown is the separation of glycopeptides and non-glycopeptides in the tryptic digest of r-tPA by rapid affinity and RPC. A micropellicular Con A column was used for fractionation of glycopeptides and non-glycopeptides (Fig. 9). In this experiment, various peptides in the original digest as well as the glyco- and non-glycopeptides fractions were separated off-line by RPC. As shown in Fig. 10, excellent separation of peptides was obtained in less than 15 min. The glycopeptide fraction contained a predominant peptide, eluted shortly after 8 min and a few components in trace amounts with retention times of 0.6 and 7.5–8.0 min. Although no effort was made to characterize the sugar moiety in the peptide (a retention time of 8.2 min), its absence in the non-glycopeptide map is taken as sufficient evidence for a glycopeptide. These results are in agreement with the chromatographic profiles described by Spellman[41].

CONCLUSION

Micropellicular stationary phases have particle diameters about 20 times smaller than those of conventional pellicular sorbents which played an important role in the early days of HPLC. Columns packed with micropellicular sorbents are emi-

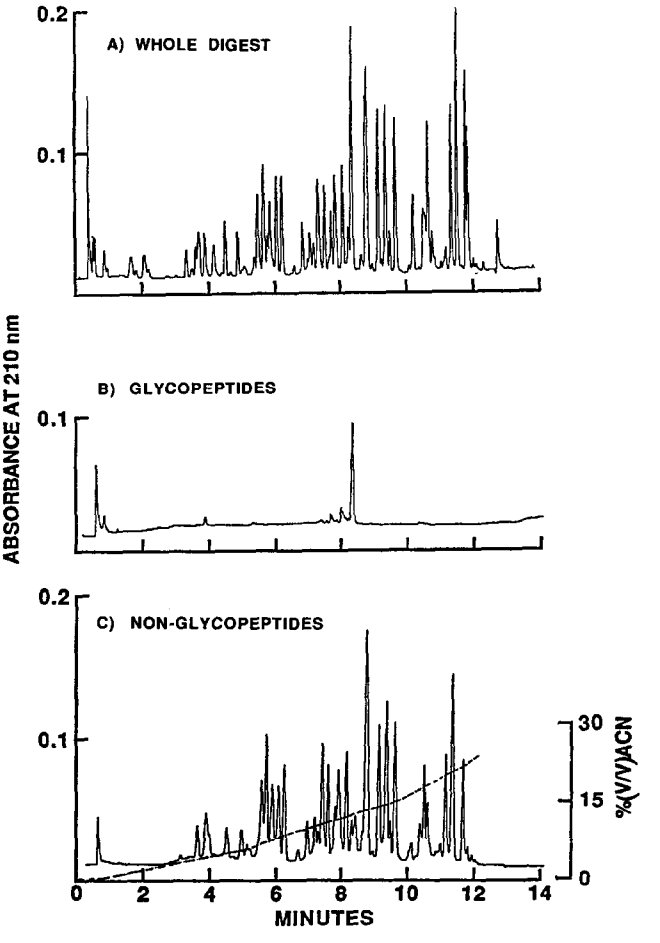


Fig. 10. Separation of tryptic fragments of r-tPA. Column, micropellicular C_{18} (75 × 4.6 mm I.D.). The flow-through fraction (non-glycopeptides) and glycopeptide fraction (Fig. 9) were freeze-dried prior to injection into the column. Eluent A, 50 m*M* phosphate buffer, containing 1 m*M* octyl sodium sulfate (pH 2.8); eluent B, 60% (v/v) acetonitrile (ACN) in 50 m*M* phosphate buffer (pH 2.8); flow-rate, 1.5 ml/min; temperature, 80°C.

nently suitable for the chromatography of proteins and offer a gain in separating speed, efficiency, stability and reproducibility comparable to that brought about by the introduction of microparticulate stationary phases in HPLC. The advantages of micropellicular sorbents demonstrated here are expected to secure for them an important place in the growing list of novel stationary phases for HPLC.

ACKNOWLEDGEMENTS

The author is indebted to Prof. Csaba Horváth for encouragement. Generous gift or r-hGH and r-tPA from Drs. W. Hancock and M. Spellman of Genentech is greatly appreciated. Contributions of Drs. G. Bonn (University of Innsbruck, Aus-

tria) and I. Fellegvári in some of the experiments are gratefully acknowledged. This work was supported by Grants GM 20993 and CA 21948 from the National Institute of Health, U.S. Department of Health and Human Services.

REFERENCES

1 W. S. Hancock, *Chromatogr. Forum*, 2 (1986) 57.
2 S. Borman, *Anal. Chem.* 59 (1987) 969A.
3 R. L. Garnick, N. J. Solli and P. A. Papa, *Anal Chem.*, 60 (1988) 2546.
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 K. K. Unger, G. Jilge, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 359 (1986) 61.
6 R. Janzen, K. K. Unger, H. Giesche, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 397 (1987) 91.
7 Y. Kato, T. Kitamura, A. Mitsui and T. Hashimoto, *J. Chromatogr.*, 398 (1987) 327.
8 K. Kalghatgi and Cs. Horváth, *J. Chromatogr.*, 398 (1987) 335.
9 K. Kalghatgi and Cs. Horváth, *J. Chromatogr.*, 443 (1988) 343.
10 L. Varady, K. Kalghatgi and Cs. Horváth, *J. Chromatogr.*, 459 (1988) 207.
11 I. Mazsaroff, M. Rounds and F. E. Regnier, *J. Chromatogr.*, 411 (1987) 452.
12 J. J. van Deemter, F. J. Zuiderweg and A. Klingenberg, *Chem. Eng. Sci.*,5 (1956) 271.
13 Cs. Horváth and H.-J. Lin, *J. Chromatogr.*, 126 (1976) 401.
14 Cs. Horváth and H.-J. Lin, *J. Chromatogr.*, 149 (1987) 43.
15 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979, pp. 168–268.
16 J. Feitlson and S. M. Partridge, *Biochem. J.*, 64 (1956) 607.
17 N. K. Boardman, *J. Chromatogr.*, 2 (1959) 388.
18 K. Randerath, *Thin Layer Chromatography*, Academic Press, New York, 2nd ed., 1956, pp. 229–243.
19 Cs. Horváth, B. Preiss and S. R. Lipsky, *Anal Chem.*, 39 (1967) 1422.
20 Cs. Horváth and S. R. Lipsky, *Anal Chem.*, 41 (1967) 1227.
21 Cs. Horváth and S. R. Lipsky, *J. Chromatogr.*, 7 (1969) 109.
22 Cs. Horváth, in J. A. Marinsky and Y. Marcus (Editors), *Ion Exchange and Solvent Extraction*, Vol. 5, Marcel Dekker, New York, 1973, pp. 207–260.
23 Cs. Horváth, in D. Glick (Editor), *Methods of Biochemical Analysis*, Vol. 21, Wiley, New York, 1973, pp. 79–154.
24 Cs. Horváth, in E. Grushka (Editor), *Bonded Stationary Phases in Chromatography*, Ann Arbor Science Publishers, Ann Arbor, MI, 1974, p. 59.
25 J. J. Kirkland, *J. Chromatogr. Sci.*, 7 (1969) 361.
26 J. J. Kirkland, *J. Chromatogr. Sci.*, 8 (1970) 72.
27 Cs. Horváth and J. M. Engasser, *Ind. Eng. Chem. Fundam.*, 12 (1973) 229.
28 Cs. Horváth, *Biochim. Biophys. Acta*, 358 (1974) 164.
29 B. Anspach, K. K. Unger, H. Giesche and M. T. W. Hearn, *Proceedings of the 4th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Baltimore, MD, December 1984*, paper 103.
30 K. K. Unger, G. Jilge, R. Janzen, J. Giesche and J. N. Kinkel, *Chromatographia*, 22 (1986) 379.
31 L. F. Colwell and R. A. Hartwick, *J. Liq. Chromatogr.*, 10 (1987) 2721.
32 D. P. Lee, *J. Chromatogr.*, 443 (1987) 71.
33 Y.-F. Maa and Cs. Horváth, *J. Chromatogr.*, 445 (1987) 71.
34 F. E. Regnier and R. Noel, *J. Chromatogr. Sci.*, 14 (1976) 316.
35 K. Kalghatgi and Cs. Horváth, in B. Wittman-Liebold (Editor), *Methods in Protein Sequence Analysis*, Springer Verlag, Berlin, 1989, p. 248.
36 M. W. Dong, J. R. Gant and B. R. Larsen, *Biochromatography*, 4 (1989) 19.
37 G. Rozing and H. Goetz, *J. Chromatogr.*, 476 (1989) 3.
38 F. D. Antia and Cs. Horváth, *J. Chromatogr.*, 435 (1988) 1.
39 G. D. Longmore and H. Schatcher, *Carbohydr. Res.*, 100 (1982), 365.
40 C. Ceccarini, P. Lorernzoni and P. H. Atkinson, *Biochim. Biophys. Acta*, 759 (1983) 214.
41 M. Spellman, in B. Karger and F. Regnier (Editors), *Proceedings of the Intensive Seminars in Analytical Biotechnology*, Bioseparations, Baltimore, MD, 1987, paper 4.