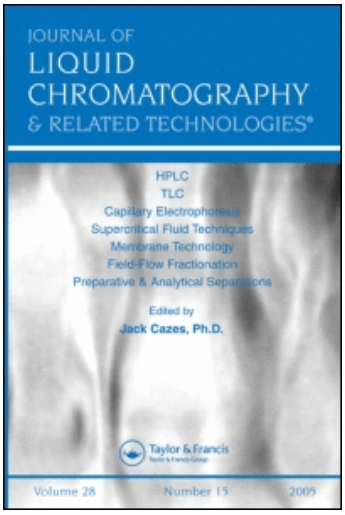

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High Performance Liquid Chromatography of Small and Large Molecules with Nonporous Silica-Based Stationary Phases

Jim Yu[ab]; Ziad El Rassi[a]

[a] Department of Chemistry, Oklahoma State University, Stillwater, OK [b] Laboratory of Biology of Addictive Diseases, The Rockefeller University, New York, NY, USA

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF SMALL AND LARGE MOLECULES WITH NONPOROUS SILICA-BASED STATIONARY PHASES

Jim Yu,† Ziad El Rassi*

Department of Chemistry
Oklahoma State University
Stillwater, OK 74078-3071

ABSTRACT

Nonporous monodispersed, microspherical silica particles, having mean particle diameters of 0.8 and 1.1 μm, were surface-modified with octadecyl or triphenyl functions as well as with lectin proteins for high performance liquid chromatography of small and large molecules. "Monomeric" or "polymeric" octadecyl-silica (C_{18}-silica) bonded phases were formed, depending on the silane compounds used. Monomeric C_{18}-silica stationary phases yielded high resolution in the separation of 2-pyridylamino derivatives of xyloglucan oligosaccharides and proteins. The effect of particle size of the support on resolution was also examined. For comparative study, the chromatographic behavior of large molecular weight proteins on a triphenyl-silica stationary phase was also conducted. Polymeric C_{18}-silica stationary phases yielded higher phase ratios and better support surface coverage which proved useful in the separation of small hydrophilic species such as derivatized chitooligosaccharides of lower degree of polymerization (d.p.).

The slopes of the linear dependence of logarithmic retention factor on volume percent of organic modifier were determined for the monomeric and polymeric C_{18}-silica stationary phases with low molecular weight aromatic compounds. Lectin affinity stationary phases with *lens culinaris* agglutinin and wheat germ agglutinin covalently bound to nonporous silica support were examined for the applications of nonporous stationary phases in lectin affinity chromatography of glycoproteins. In all cases, rapid separations in the time scales of seconds and minutes could be obtained because of the absence of mass transfer resistance in the nonporous stationary phase matrices.

INTRODUCTION

The development of rapid separation schemes by HPLC has been an important theme of research in recent years. This is because fast chromatographic separations ensure short analysis time, rapid development of analytical methods, high mass recovery with preserved bioactivity for biological species, and improved separation efficiency.

Mass transfer resistances and solute adsorption-desorption kinetics are the two main factors that influence band broadening in chromatography. To reduce the mass-transfer resistance arising from slow diffusion commonly encountered in traditional porous column packing materials, one approach is to use particles with flow-through macropores.[1] Another approach is to eliminate the pore structure by using nonporous packing. Nonporous silica packing of small particle diameter (0.5 - 2.0 μm) offers about the same specific surface area per unit column volume as a silica with 400 nm pore size, 5 to 10 μm particle diameter and a packing density of 0.5 g/mL.[2]

Columns packed with nonporous particles have been used for rapid separation of biomacromolecules, such as peptides, proteins, oligonucleotides and nucleic acid restriction fragments, by reversed phase,[3-5] ion exchange[6,7] and hydrophobic interaction chromatography.[8] Furthermore, the application of nonporous silica and zirconia packing materials for reversed phase chromatographic separation of low-molecular-weight compounds was recently investigated.[9-11]

This paper reports the applications of nonporous monodispersed, microspherical silica particles having mean particle diameter of 0.8 and 1.1 μm. These particles were modified with octadecyl or triphenyl functions to

yield nonpolar stationary phases, and with lectins (e.g., LCA and WGA) to produce lectin affinity stationary phases. The chromatographic properties of the various modifications on these nonporous supports were evaluated with small and large molecular weight compounds.

EXPERIMENTAL

Instrumentation

The liquid chromatograph was assembled from (i) an LDC Analytical (Riviera Beach, FL, U.S.A.) ConstaMetric 3500 solvent delivery system with a gradient programmer, which was used to control a ConstaMetric Model III solvent delivery pump; (ii) a sample injector Model 7125 from Rheodyne (Cotati, CA, U.S.A.); and (iii) a UV interference filter photometric detector Model UV-106 from Linear Instruments (Reno, NV, U.S.A.). Chromatograms were recorded with a computing integrator Model C-R6A equipped with a floppy disk drive and a cathode-ray tube (CRT) monitor from Shimadzu (Columbia, MD, U.S.A.).

Reagents and Materials

HPLC grade acetonitrile and methanol, reagent grade sodium phosphate mono- and di-basic, sodium hydroxide, sodium chloride, sucrose, glacial acetic acid, anhydrous ammonia, saturated ammonium hydroxide, trifluoroacetic acid (TFA), benzene, toluene, *p*-xylene, and naphthalene, and reagent grade as well as technical grade isopropanol, methanol, 200 proof denatured ethanol, and *N, N*-dimethylformamide (DMF) were from Fisher (Pittsburgh, PA, U.S.A.). Tetraethylorthosilicate (TEOS), trimethylchlorosilane, sodium cyanoborohydride and sodium periodate were purchased from Aldrich (Milwaukee, WI, U.S.A.). γ-Glycidoxypropyltrimethoxysilane, triphenylchlorosilane, octadecyldimethylchlorosilane and octadecyltrichlorosilane were obtained from Hüls America Inc. (Bristol, PA, U.S.A.).

Ribonuclease A from bovine pancreas, cytochrome c from horse heart, lysozyme from chicken egg, glucose oxidase from *Aspergillus niger*, ovalbumin, α_1-acid glycoprotein from bovine and human, *p*-nitrophenyl-*N*-acetyl-α-D- and β-D-glucosaminide, *p*-nitrophenyl-*N*-acetyl-β-D-*N,N'*-diacetylchitobioside were purchased from Sigma (St. Louis, MO, U.S.A.). *N*-acetyl-D-glucosamine, *N*-acetyl-D-chitobiose, *N*-acetyl-D-chitotriose, and *N*-

acetyl-D-chitotetraose were obtained from Seikagaku America, Inc. (Rockville, MD, U.S.A.) and were labeled with 2-aminopyridine using a well established procedure.[12] *Lens culinaris* agglutinin (LCA) and wheat germ agglutinin (WGA) were from Vector Laboratories, Inc. (Burlingame, CA, U.S.A.).

Silica Supports

Microspherical, monodispersed nonporous silica particles were prepared by seed-growth process of base-catalyzed hydrolysis of TEOS following the procedures described earlier.[10] Briefly, 500 mL of ethanol placed in a 1.0 L three-neck round bottom flask was first saturated with anhydrous ammonia by bubbling it through the solution. Thereafter, 127 mL of saturated ammonium hydroxide and 8.0 mL TEOS were added while stirring. After 10 h, addition of 6.0 mL of TEOS and 1.4 mL water were repeated at every 12 h interval for a total of six additions. Silica particles thus prepared have a nonporous texture, monodispersed spherical shape with a mean diameter of *ca.* 0.8 μm. Nonporous monodispersed silica particles in the size of 1.1 μm were prepared as the above procedure except that 16 mL TEOS and 1.4 mL water were added for a total of five additions.

Commercially available Nucleosil silica of 7 μm mean particle diameter and 300 Å mean pore diameter was obtained from Machery-Nagel (Düren, Germany), and used as supplied.

Stationary Phases

Octadecyl-silica stationary phases

Monomeric and polymeric C_{18}-silica stationary phases were synthesized according to the procedures described in an earlier study.[10] The silica microspheres were treated with octadecyldimethylchlorosilane to form "monomeric" stationary phase. Further treatment of some monomeric C_{18}-silica stationary phases with chlorotrimethylsilane was performed to end-cap the residual silanol groups that might remain unreacted.

Octadecyl-silica of the "polymeric" type was formed by reacting the silica with octadecyltrichlorosilane. Both modifications were carried out in toluene while refluxing at 120°C for 12 h. End-capping with chlorotrimethylsilane was carried out in toluene at 60°C for 12 h.

Triphenyl-silica stationary phase

Typically, 3.0 g of silica support were suspended in 30.0 mL of toluene, and heated to 120°C. Then 8.0 mL of triphenylchlorosilane were added to the solution. The reaction mixture was stirred for 12 h at 120°C. After the reaction, the silica thus treated was separated from the solution, and then washed successively with toluene and methanol, and let dry in the air.

Lectin stationary phases

Microspherical silica particles were first epoxy activated by reacting them with γ-glycidoxypropyltrimethoxysilane in 1.0 mM Na_2HPO_4, pH 6.0. The reaction mixture was stirred at 95°C for 4 h. Thereafter, the epoxy ring was opened with 0.01 M HCl at 90°C, and then converted to aldehyde by allowing the diol-silica phase to react with sodium periodate in glacial acetic acid-water (9:1, v/v) at room temperature. The aldehyde-activated silica microparticles were reacted with WGA or LCA. In both cases, 50 mM phosphate buffer, pH 6.0, containing sodium cyanoborohydride was used as the reaction medium.

Column Packing

All columns, 3.0 x 0.46 cm I.D. No. 316 stainless steel tubes (Alltech Associates Inc., Deerfield, IL, U.S.A.) were packed at 7000 *psi* using slurry packing technique with Shandon column packer instrument (Keystone Scientific, Bellefonte, PA, U.S.A.). Octadecyl-silica stationary phases were packed from an isopropanol slurry with isopropanol as packing solvent. Lectin stationary phases were packed from an aqueous sucrose-NaCl slurry containing 50% (w/v) sucrose, with 1.0 M NaCl as the packing solvent.

RESULTS AND DISCUSSION

Reversed-Phase Chromatography

Reversed-phase chromatographic properties

Benzene, toluene, *p*-xylene and naphthalene were employed as model solutes to evaluate the chromatographic properties of the nonporous C_{18}-silica of 0.8 μm average particle diameter using mobile phases composed of water-acetonitrile mixtures at various volume percent of acetonitrile. As shown in Figure 1A and 1B, plots of log k' *vs.* φ are linear and follow the expression usually found in RPC:[13]

$$\log k' = \log k_w - S\varphi \quad (1)$$

where S is the slope of the linear function, which is characteristic of the solute molecular weight for a given stationary phase, φ is the volume fraction of the organic solvent in the mobile phase, and k_W is the retention factor obtained with pure water. The value of S for a given solute has important consequences in the selection of optimum gradient elution conditions.[14] For porous stationary phases, and to a first approximation, S is given by[15]

$$S = 0.48M^{0.44} \quad (2)$$

where M is the molecular weight of the solute. The S values of the four aromatic solutes obtained on the monomeric and polymeric C_{18}-silica stationary phases are listed in Table 1. As can be seen in Table 1, the S values for the solutes obtained on both types of C_{18}-silica stationary phases increased with increasing molecular weight of the solute. Compared to the calculated S values using equation (2), which applies to porous packings, the S values obtained on the nonporous silica sorbent are in the same range, except for that of benzene. Conversely, the S values of large molecular weight proteins (MW in the range 12,000-162,000) were reported to be 10-fold smaller on nonporous than on porous support.[3] This may indicate that the rapid mass transfer of large molecules on the external surface of the packing affects the rate constants of adsorption and desorption in such a way that lower equilibrium constants and distribution coefficients will result.[3] For small molecules the difference in kinetics of the diffusion process will be much less significant, when comparing solute diffusion into and out of a pore system to solute diffusion on exclusively external surfaces. This may explain why the S values for small molecules obtained on nonporous ODS packings were similar to the calculated values.

Chromatographic behavior of 2-AP derivatized linear and branched oligosaccharides

In our previous studies,[10,11] it was found that polymeric C_{18}-silica stationary phases exhibit higher phase ratios and better support surface coverage than the monomeric ones. This was especially important in the separations of small molecules of closely related species, *e.g.* dansyl amino acids. Similarly, and because of its relatively high surface density in octadecyl function, the polymeric C_{18}-silica stationary phase was useful for the separation of hydrophilic species such as the 2-pyridylamino derivatives of chitooligosaccharides of low d.p. (see Figure 2). As can be seen in Figure 2, the four solutes could be separated in less than four minutes with gradient elution of up to 10% (v/v) acetonitrile in the mobile phase at a flow rate of 2.0 mL/min.

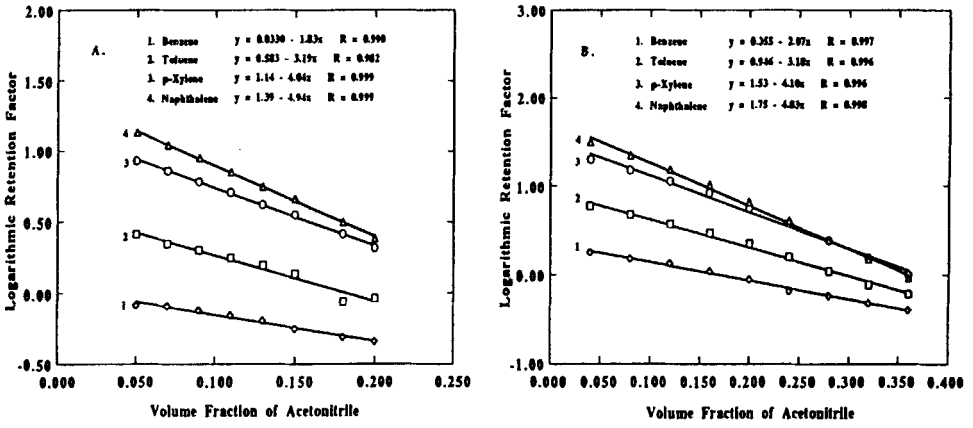

Figure 1. Plots of logarithmic retention factor versus the volume percent acetonitrile in mobile phase for end-capped monomeric octadecyl-silica stationary phase in A; and polymeric octadecyl-silica stationary phase in B. Columns, 30.0 x 4.6 mm; mobile phase, water at various volume percent acetonitrile; flow rate, 2.0 mL/min.

Table 1

Values of S Calculated using Equation (2) and Determined from the Linear Plots According to Equation (1)

		S-Value		
	M.W.	Calculated	Monomeric C_{18}-silica	Polymeric C_{18}-silica
Benzene	78.11	3.27	1.83	2.07
Toluene	92.14	3.51	3.19	3.18
p-Xylene	106.17	3.74	4.04	4.10
Naphthalene	128.17	4.06	4.94	4.83

Column, 30.00 x 4.6 mm, end-capped monomeric and polymeric octadecyl-silica stationary phases; mobile phase, water at various volume fraction of acetonitrile; flow rate, 2.0 mL/min.

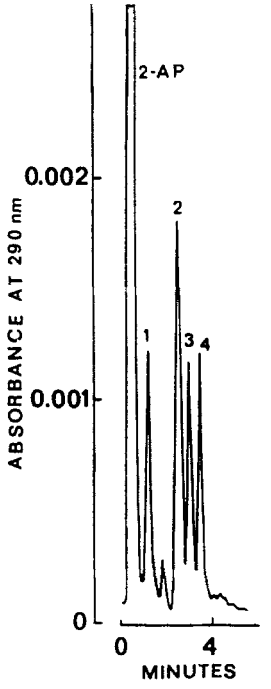

Figure 2. Chromatograms of 2-pyridylamino derivatives of chitooligosaccharides obtained on polymeric octadecyl-silica stationary phase. Column, 30.0 x 4.6 mm; linear gradient in 5.0 min from 0.0 to 10.0% (v/v) acetonitrile in 10.0 mM NaH_2PO_4, pH 3.0; flow rate, 2.0 mL/min; samples: 2-pyridylamino derivatives of 1, *N*-acetyl-D-glucosamine; 2, chitobiose; 3, chitotriose; 4, chitotetraose; detection, UV, l = 290 nm.

On the other hand, for branched oligosaccharides such as 2-pyridylamino derivatives of xyloglucan oligosaccharides, monomeric C_{18}-silica columns yielded better separation than polymeric C_{18}-silica stationary phases. Figure 3 illustrates the various maps of 2-pyridylamino derivatives of xyloglucan oligosaccharides (PA-XG) at different mobile phase flow rates and gradient duration, performed on a short column (30.0 x 4.6 mm) packed with 0.8 μm nonporous octadecyl-silica monomeric stationary phase. As can be seen in the chromatograms, upon increasing the flow rate from 1.0 mL/min to 3.0 mL/min and gradient steepness from 0.40 to 0.85% (v/v) acetonitrile/min, the analytical information about the content of the mixture remained almost unchanged, as far as the larger PA-XG fragments (*i.e.* retarded peaks) are concerned. In addition, the peaks were sharper at the mobile phase flow rate of 3.0 mL/min than at 1.0 mL/min, and, consequently, for the same signal the amount injected

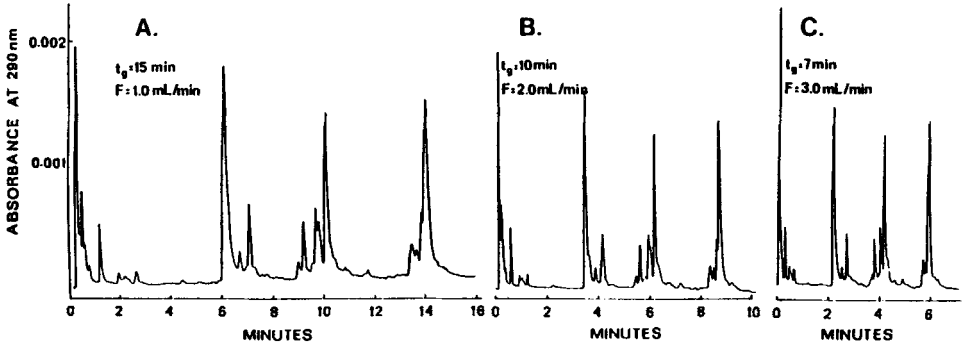

Figure 3. Chromatograms of 2-pyridylamino derivatives of xyloglucan oligosaccharides obtained on end-capped monomeric octadecyl-silica (d_p = 0.8 μm) stationary phase. Column, 30.0 x 4.6 mm; linear gradient from 0.0 to 6.0% (v/v) acetonitrile in 10.0 mM NaH_2PO_4, pH 3.0; the gradient duration (t_g) and flow rate (F) are indicated in each panel; detection, UV, l = 290 nm.

was 2 to 3 times lower at higher flow velocity. This clearly demonstrates that rapid reversed phase chromatography with appropriately designed nonporous octadecyl-silica stationary phase yields excellent separation for closely related oligosaccharides with high sensitivity and high separation efficiencies.

When the same oligosaccharide mixture was chromatographed on the same size silica gel (*i.e.*, 0.8 μm) but with "polymeric" octadecyl bonded phase, the amount of organic solvent needed to bring about the same time of analysis as with the "monomeric" octadecyl-silica packings is higher (*ca. 1.7*-fold, results not shown). This is expected as the phase ratio is increased. However, the resolution did not improve when going from monomeric to polymeric octadecyl bonded phase, and part of the analytical information about the mixture was lost, even when using the same gradient profile as with the monomeric octadecyl sorbent. This corroborates our previous findings[16] with porous octadecyl silica stationary phases in that higher phase ratio sorbents, which necessitates increased percent of organic solvent in the mobile phase, did not bring about good resolution for this mixture. This is probably due to organic solvent-induced conformational changes of the analytes. In higher organic solvent mobile phases, the difference in the molecular hydrophobic surface areas of the various sugar chains was diminished during the elution process of the oligomers from the stationary phase, and as a result lower selectivity was obtained.

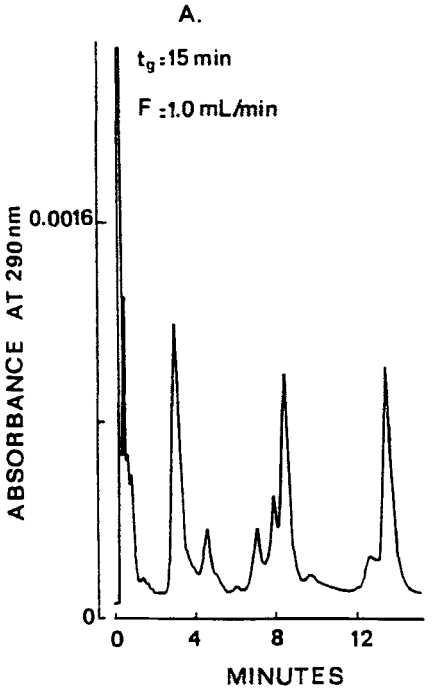

Figure 4. Chromatograms of 2-pyridylamino derivatives of xyloglucan oligosaccharides obtained on end-capped monomeric octadecyl-silica (d_p = 1.1 μm) stationary phase. Column, 30.0 x 4.6 mm; linear gradient from 0.0 to 6.0% (v/v) acetonitrile in 10.0 mM NaH_2PO_4, pH 3.0; the gradient duration (t_g) and flow rate (F) are indicated in the figure; detection, UV, l = 290 nm.

To determine the effect of particle size on the resolution of the oligomers, the PA-XG oligosaccharides were chromatographed on a 1.1 μm nonporous silica gel having monomeric octadecyl bonded phase. The results are shown in Figure 4. As expected, increasing the particle size by a factor of 1.4 resulted in decreased retentivity of the column as a result of the decreased specific surface area of the silica particles. The percent organic solvent in the mobile phase needed to bring about the same time of analysis as with 0.8 μm decreased by a factor of 1.5. Also, the resolution for the early eluting peaks decreased significantly, and part of the analytical information was lost. It seems that there is an optimum phase ratio that is best attained by decreasing the particle size of the support and using monomeric octadecyl bonded stationary phase for the rapid and high efficiency separation of these branched oligosaccharides.

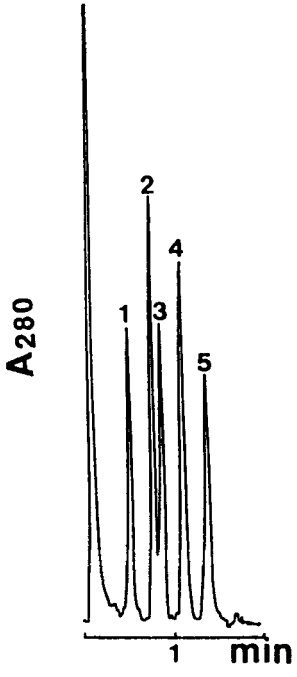

Figure 5. Chromatogram of proteins obtained on monomeric octadecyl-silica stationary phase without end-capping. Column, 30.0 x 4.6 mm; linear gradient in 2.5 min from 20.0 to 70.0% (v/v) acetonitrile in aqueous mobile phase with 0.05% (v/v) TFA; flow rate, 4.0 mL/min; samples: 1, ribonuclease A; 2, cytochrome c; 3, lysozyme; 4, albumin (bovine); 5, ovalbumin; detection, UV, l = 280 nm.

Chromatographic behavior of proteins

Although the low phase ratio of monomeric C_{18}-silica stationary phase on 1.1 μm silica support limits the resolution of hydrophilic species (see preceding section), this feature, on the other hand, can be regarded as an advantage in terms of bringing chromatographic retention to a practical range, especially in the chromatography of biomacromolecules, *e.g.*, proteins. Under these circumstances, biopolymers may be separated under milder elution conditions which would preserve their biological activity and allow their high mass recovery. Figure 5 is an example of the high speed separation of a group of five proteins on monomeric C_{18}-silica stationary phase without end-capping having a mean particle diameter of 1.1 μm.

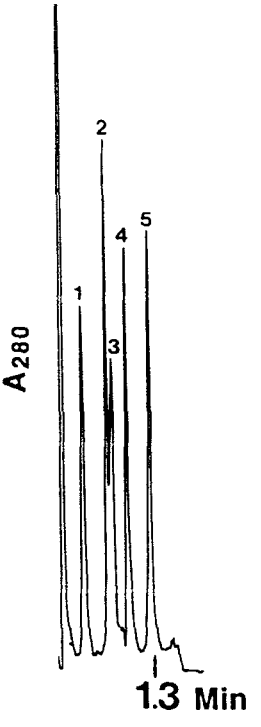

Figure 6. Chromatogram of proteins obtained on triphenyl-silica stationary phase. Column, 30.0 x 4.6 mm; linear gradient in 1.5 min from 20.0 to 70.0% (v/v) acetonitrile in aqueous mobile phase with 20.0 mM NaCl and 0.05% (v/v) TFA; flow rate, 4.0 mL/min; samples: 1, ribonuclease A; 2, cytochrome c; 3, lysozyme; 4, albumin (bovine); 5, ovalbumin; detection, UV, l = 280 nm.

The separation was accomplished in less than 1.5 min at mobile phase flow rate of 4.0 mL/min using a gradient elution from 20 to 70% (v/v) acetonitrile in the mobile phase in 2.5 min. Even though the phase ratio for this stationary phase was quite low, its application for the separation of macromolecules could still produce enough resolving power.

Silica microparticles (1.1 μm) were also modified with triphenyl functional groups. The same group of proteins chromatographed on the C_{18}-silica stationary phase was separated on the triphenyl-silica stationary phase, see Figure 6. The elution pattern was the same, except that 20.0 mM NaCl had to be added to the mobile phase since otherwise these proteins could not be eluted from the triphenyl stationary phase. This may indicate the presence of

unreacted surface silanols which act as cation exchange sites for polyionic species such as proteins. When the same hydro-organic mobile phase was used for both C_{18}-silica and triphenyl-silica stationary phases, slightly lower retention for all the proteins was observed on the triphenyl phase, possibly because of the lower hydrophobicity of the triphenyl functions when compared to the octadecyl functions. Better resolution between cytochrome c and lysozyme was obtained on the C_{18}-silica than on the triphenyl-silica stationary phase (compare Figures 5 and 6).

Lectin Affinity Chromatography of Glycoproteins and Small Glycoconjugates

Glycoproteins are heterogeneous species due to the variations in the oligosaccharide chains attached.[17] This heterogeneity is amplified when the glycoprotein solute is multiply glycosylated with variations in the number and location of the oligosaccharides as well as in the nature of the sugar chains. These microheterogeneities lead to multiple forms for a given glycoprotein, and they are referred to as glycoforms. These glycoforms have been shown to correlate with certain clinical syndromes.[18,19] Thus, the ability to separate and quantitatively measure the various glycoforms of a given glycoprotein has become increasingly important. In this regard, lectins which are sugar-binding proteins[20] are powerful tools for studying glycoprotein microheterogeneity.[21,22] In particular, lectin affinity chromatography can reveal the microheterogeneity of glycoproteins, imparted by their oligosaccharide chains. This is often manifested by the presence of three different peaks:

(i) non-reactive population of the molecules, *i.e.*, molecules missing the oligosaccharide moieties that can be recognized by the lectin, which are eluted in the void volume of the column with the binding buffer;

(ii) weakly reactive population of the molecules, *i.e.*, molecules containing weakly reactive oligosaccharide chains; and

(iii) strongly reactive molecules which are specifically desorbed and eluted with the debinding buffer containing the appropriate haptenic sugar.[17]

Two lectins, LCA and WGA, were immobilized onto nonporous silica microparticles for rapid lectin affinity chromatography of glycoconjugates. Figure 7A and B show the rapid lectin affinity chromatography of glucose

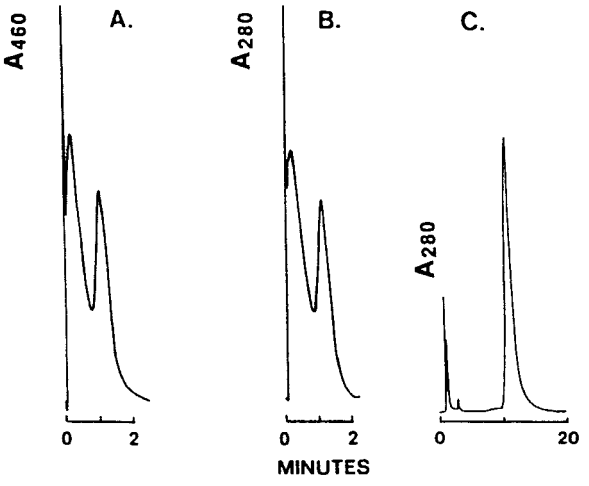

Figure 7. Chromatograms of glucose oxidase obtained on nonporous (0.8 μm) silica-bound LCA stationary phases in (A) and (B), and porous (7 mm, 300 Å) silica-bound LCA stationary phase in (C). (A) and (B): column, 30.0 x 4.6 mm; gradient from 0.0 to 25.0 mM methyl-α-D-mannopyranoside in 25.0 mM Na_2HPO_4, pH 6.0, and 200.0 mM NaCl, in 0.20 min, and isocratic elution for 2.0 min; flow rate, 3.0 mL/min; (C): column, 100.0 x 4.6 mm; linear gradient from 0.0 to 20.0 mM methyl-α-D-mannopyranoside in 20.0 mM $NaOOCCH_3$, pH 6.0, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 200.0 mM NaCl, in 1.0 min, and isocratic elution for 10.0 min; flow rate, 1.0 mL/min. Detection, UV, (A) l = 460 nm, (B) and (C) l = 280 nm.

oxidase performed on a short column (30.0 x 4.6 mm) packed with 0.8 μm nonporous LCA-silica. As can be seen in Fig. 7, the LCA column yielded three peaks for the protein. One peak eluted in the void volume of the column, and is believed to correspond to the non-reactive population of the glycoprotein molecules. A second peak corresponding to molecules missing the saccharide determinant, *i.e.*, weakly reactive components, was slightly retained, and eluted with the binding buffer. The third peak was specifically eluted with the debinding buffer containing the haptenic sugar, methyl-α-D-mannopyranoside, thus corresponding to the molecules containing the oligosaccharide chains with the sequences that are necessary for lectin recognition, *i.e.*, strongly reactive components. Note that glucose oxidase is a flavoprotein, the prosthetic group being flavin adenine dinucleotide which is well detected in the visible region at 460 nm (see Figure 7A); the chromatogram recorded at 280 nm corresponds to the specific UV absorbance of the peptide bonds of the protein. On the other hand, on porous silica (300 Å) with surface bound LCA, the weakly binding

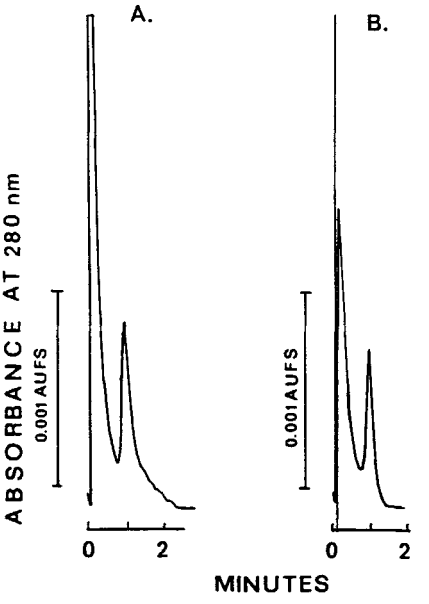

Figure 8. Chromatograms of α_1-acid glycoprotein from bovine in A and human in B obtained on nonporous silica-bound WGA stationary phase. Column, 30.0 x 4.6 mm; gradient from 0.0 to 25.0 mM *N*-acetylglucosamine in 25.0 mM Na_2HPO_4, pH 6.0, and 200.0 mM NaCl, in 0.20 min, and isocratic elution for 2.0 min; flow rate, 3.0 mL/min.

fraction of glucose oxidase (i.e., the second peak with the nonporous LCA column) seemed to elute with the lectin reactive fraction, and one single retained peak was recorded, see Figure 7C. This can be explained by the much higher phase ratio obtained on the porous support. The strong binding interactions exhibited by the highly populated lectin stationary phase rendered the two fractions of glucose oxidase to coelute at the same concentration of methyl-α-D-mannopyranoside in the eluent using gradient elution.

Figure 8 illustrates the rapid lectin affinity chromatography of bovine and human α_1-acid glycoproteins obtained on a column packed with 1.1 μm nonporous WGA-silica particles. The selectivity of the lectin column permitted the illustration of the microheterogeneity of the glycoproteins. α_1-Acid glycoprotein from bovine was separated into two fractions, see Figure 8A. One fraction eluted first, and is believed to contain the non-reactive and slightly reactive components of the glycoprotein. The second peak containing the strongly reactive components was highly retained and eluted with the haptenic

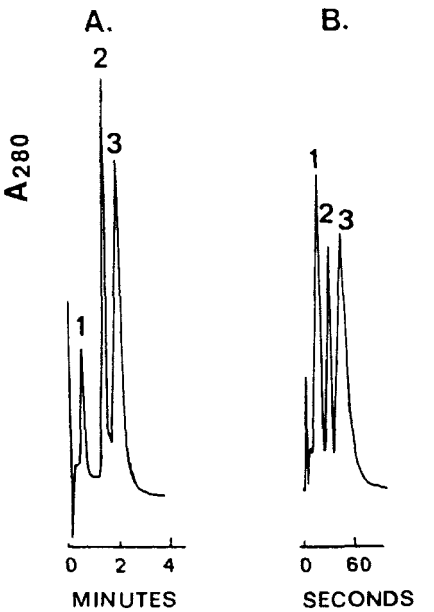

Figure 9. Chromatograms of *p*-nitrophenyl derivatives of saccharides obtained on nonporous silica-bound WGA stationary phase. Column, 30.0 x 4.6 mm; in A linear gradient from 0.0 to 50.0 mM *N*-acetylglucosamine in 100.0 mM Na_2HPO_4, pH 6.0, in 1.0 min, and isocratic elution for 2.0 min; in B linear gradient from 0.0 to 100.0 mM *N*-acetylglucosamine in 100.0 mM Na_2HPO_4, pH 6.0, in 0.50 min, and isocratic elution for 2.0 min; flow rate, 3.0 mL/min in both cases; samples, *p*-nitrophenyl derivatives of 1, *N*-acetyl-α-D-glucosaminide; 2, *N*-acetyl-β-D-glucosaminide; 3, b-D-*N,N'*-diacetylchitobioside; detection, UV, l = 280 nm.

sugar. α_1-Acid glycoprotein from human yielded three peaks (Figure 8B): one non-reactive, another slightly retained containing the weakly reactive components, and the third one was retarded containing the strongly reactive components.

Lectin affinity chromatography is characterized by its inherent slow sorption kinetics which often leads to bandspreading. With porous particles, axial dispersion at high flow velocities would be influenced by two independent processes, kinetic resistance and intraparticular diffusion resistance. With nonporous affinity stationary phases, however, since there is no solute mass transfer in and out of the pores, the major contribution to bandspreading comes only from the sorption kinetics. A more energetically uniform surface is

expected to result from the modification of support having totally exposed surface of nonporous texture than from the modification of porous sorbents. As a consequence, nonporous stationary phases may exhibit the sorption kinetics that are significantly faster than that of the porous stationary phases. The high efficiency of nonporous WGA-silica stationary phase was demonstrated with the rapid separation of *p*-nitrophenyl derivatives of mono- and disaccharides (see Figure 9). The high resolution was achieved even when steep gradient of strong eluent was used, and the separation was accomplished in less than 1min.

CONCLUSIONS

The chromatographic applications of nonporous monodispersed, microspherical silica particles were examined for rapid HPLC. Surface modification with octadecyl functions for the formation of reversed phases have shown that the polymeric C_{18}-silica stationary phases are suitable for the separation of derivatized homooligosaccharides of low d.p. (e.g., 2-pyridylamino-chitooligosaccharides). The monomeric C_{18}-silica, when the surface modification was properly carried out, can yield higher resolving power than the polymeric type toward derivatized branched heterooligosaccharides. Uniformly covered supports of low ligand density, such as monomeric C_{18}-silica without end-capping, are suitable for high-speed separation of biomacromolecules such as proteins. On the other hand, low ligand density silica bonded stationary phases having covalently bound triphenyl functions necessitated the addition of small amounts of sodium chloride to the mobile phase to bring about the elution and separation of proteins. The formations of lectin affinity stationary phases on the nonporous silica supports have proved to be useful for the rapid analysis of the microheterogeneity of glycoproteins.

REFERENCES

1. N. B. Afeyan, S. P. Fulton, F. E. Regnier, J. Chromatogr., **544**, 267-279 (1991).

2. K. K.Unger, G. Jilge, R. Janzen, H. Giesche, J. N. Kinkel, Chromatographia, **22**, 379-380 (1986).

3. K. K. Unger, G. Jigle, J. N. Kinkel, M. T. W. Hearn, J. Chromatogr., **359**, 61-72 (1986).

4. K. Khalghatgi, Cs. Horváth, J. Chromatogr., **443**, 343-354 (1988).

5. Y.-F. Maa, Cs. Horváth, J. Chromatogr., **445**, 71-86 (1988).

6. Y. Kato, T. Kitamura, A. Mitsui, T. Hashimoto, J. Chromatogr., **447**, 212-220 (1988).

7. Y. Kato, Y. Yamasaki, A. Onaka, T. Kitamura, T. Hashimoto, T. Murotsu, S. Fukushige, K. Matsubara, J. Chromatogr., **478**, 264-268 (1989).

8. R. Janzen, K. K. Unger, H. Giesche, J. N. Kinkel, M. T. W. Hearn, J. Chromatogr., **397**, 91-97, (1987).

9. H. Itoh, T. Kinoshita, N. Nimura, J. Liq. Chromatogr., **16**, 809-823 (1993).

10. J. Yu, Z. El Rassi, J. Liq. Chromatogr., **16**, 2931-2959 (1993).

11. J. Yu, Z. El Rassi, J. Chromatogr., **631**, 91-106 (1993).

12. W. Nashabeh, Z. El Rassi, J. Chromatogr., **536**, 31-42 (1991).

13. L. R. Snyder, "Gradient Elution," in **High-Performance Liquid Chromatography**, Cs. Horvath, ed., Vol. 1, Academic Press, New York, 1980, p. 207-316.

14. L. R. Snyder, J. L. Glajch, J. J. Kirkland, "Gradient Elution," in **Practical HPLC Method Development**, Wiley-Interscience, New York, 1988, pp. 153-178.

15. M. A. Stadalius, H. S. Gold, L. R. Snyder, J. Chromatogr., **296**, 31-59 (1985).

16. Z. El Rassi, D. Tedford, J. An. A. Mort, Carbohydr. Res., **215**, 25-38 (1991).

17. J. Montreuil, S. Bouquelet, H. Debray, B. Fournet, G. Spik, G. Strecker, "Glycoproteins," in **Carbohydrate Analysis**, M. F. Chaplin and J. F. Kennedy, eds., IRL Press, Oxford, 1986, pp. 143-204.

18. H. G. van Ejik, W. L. van Noort, G. de Jong, J. F. Koster, Clin. Chim. Acta, **165**, 141-145 (1987).

19. H. Stibler, O. Sydow, S. van der Berg, Pharmacol. Biochem. Behav., **13**, 47-51 (1980).

20. H. Debray, J. Montreuil, "Specificity of Lectins Toward Oligosaccharidic Sequences Belonging to *N*- and *O*-Glycosylglycoproteins," in **Affinity Electrophoresis, Principles and Application**, J. Breborowicz, A. Mackiewicz eds., CRC Press, Boca Raton, 1992, pp. 23-57.

21. H. Lis, N. Sharon, "Lectins: Their Chemistry and Application to Immunology," in **The Antigens**, M. Sela, ed., Vol. 4, Academic Press, New York, 1977, pp. 429-529.

22. I. Goldstein, C. Hayes, Adv. Carbohydr. Chem. Biochem., **35**, 127-340 (1978).

† Current affiliation: Laboratory of Biology of Addictive Diseases, The Rockefeller University, New York, NY 10021-6399, USA.

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