Ultrafast Reversed-Phase High-Performance Liquid Chromatographic Separations: An Overview

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Abstract

Ultrafast reversed-phase high-performance liquid chromatographic (HPLC) separations are often needed for analyses related to combinatorial chemistry, studies in liquid chromatography–mass spectrometry, and other applications in which very rapid sample turnaround is paramount. Unfortunately, no consensus exists regarding the best column technology for optimally performing the desired rapid separations. This overview compares the advantages and limitations for columns of ultramicroporous, ultramicrononporous, and superficially porous particles and monolith structures for the very fast separation of solutes by reversed-phase HPLC. Data from literature and the author's laboratory are used to illustrate the strengths and limitations of the various approaches that can be used for ultrafast separations.

Introduction

Chromatographic theory clearly predicts that high-performance liquid chromatographic (HPLC) separations can be made more efficient by using columns with smaller diameter particles (1). In recent years, practitioners have increasingly used smaller particles mainly to decrease the time required to perform a needed separation. Many laboratories are now routinely using columns of 3 to 3.5-µm particles rather than the more conventional 5-µm particles in order to gain the same separation in approximately half the separation time (2). Further improvements in resolution and separation time can be obtained by using porous or nonporous particles of less than 3 µm, but at a sacrifice of decreased operational convenience (3–5). Columns composed of in-place-synthesized monolith silica (6) and organic polymer (7) materials have also demonstrated rapid separations. Another particle configuration used for very fast separations is based on superficially porous (SP) particles (8,9). These particles (typically around 5 µm in diameter) have a solid core with an outer porous shell containing an interactive stationary phase. The thin porous shell of these particles exhibit fast mass-transfer properties, which makes them especially useful for rapidly separating macromolecules with poor diffusional characteristics (9).

Both porous and nonporous ultramicroparticles have been studied for high-performance separations, each configuration having advantages and disadvantages for particular applications. Nonporous particles of 1 to 2 µm in diameter have received special attention because of the favorable mass transfer afforded by the thin skin-like outer stationary-phase surface. Such materials are especially proposed for rapidly separating macromolecular compounds that slowly diffuse in the mobile phase (3,4,10). However, other workers have used columns based on 1.5-µm nonporous silica particles for rapidly separating small molecules (11,12). The comparison of 1.5-µm nonporous silica and 3.5-µm porous silica particles has also been reported (13).

Columns of small nonporous particles have exhibited excellent ultrafast separations of protein mixtures (14). These particles have also been used for other high-performance separations, particularly affinity chromatography (15) and packed-column hydrodynamic chromatography (16). Columns of 2-µm pellicular ion-exchange particles used for rapidly separating protein mixtures have also been described (17). Nonporous 1.5-µm particles in packed capillary columns at ultrahigh pressures allow very high plate numbers to be generated for separating complex mixtures (18).

Porous silica particles of ≤ 2 µm have also demonstrated very rapid high-performance separations. For example, 1.5 to 2.0-µm wide-pore porous silica microspheres have been used for the fast reversed-phase separations of peptides and proteins (5,19). Smaller molecules such as antidepressant drugs have also been rapidly determined with columns of 2-µm narrow-pore particles (20). Modified 1.8-µm porous silica have also been used for high-performance separations by capillary electrochromatography (21).

Monolith columns include both large channels (which enable unrestricted rapid flowthrough of the mobile phase) and smaller pores (which provide the surface required for the separation process). The convective flow generated through the structure is apparently sufficient to substantially increase mass transfer (6,7). As a result, very rapid separations can be performed with these materials.

SP particles have been known for some time, having been commercially available in approximately 40-µm particle-diameter column packings in the late 1960s (22). However, recent studies have led to the development of SP particles in the 5-µm range,

which have led to renewed interest in this configuration for very high speed separations (8,9). A recent new look at the independent kinetic processes that take place in a packed bed has suggested that superficially porous packings may have significant promise for very fast separations based on turbulent flow conditions (23). However, applications with columns of small superficially porous particles are sparse because of the current lack of commercial availability.

Although practitioners in the above referenced studies and others have commended the use of very small particles, monoliths, and SP particles for HPLC separations, few attempts have been made to define both the advantages and limitations of these materials for practical routine separations. In some instances, the virtues of a particular particle type have been asserted by using comparative separation data that was optimized only for the proposed particles. In other studies, the virtues of using very small particles have been given but some of the disadvantages have not been discussed. This study attempts a broader perspective in the use of ultramicroparticles, monoliths, and SP particles for ultrafast HPLC separations so that the reader is aware of the advantages and limitations of these approaches. In this way, better judgments regarding the selection of column types for very fast separations might be facilitated. In a sense, this overview updates the presentation found in reference 4, except that the focus is largely on experimental rather than theoretical comparisons of the particle types.

Experimental

HPLC separations in this laboratory were performed with a Model 1100 liquid chromatograph (LC) with a multiwavelength ultraviolet (UV) detector and a microcell (Agilent Technologies, Palo Alto, CA), a Model 8125 sample injection valve (Rheodyne, Rohnert Park, CA), and a ChemStation data-handling system (Agilent Technologies). Test solutes were from Aldrich Chemical Co. (Milwaukee, WI) or Sigma (St. Louis, MO) and used as received. Zorbax Rx-SIL 1.8-µm particles were synthesized by a procedure similar to that previously described (5). These particles were bonded with sterically protecting di-isobutyl-C18 groups in a manner similar to that previously given (24) in order to form the designated Zorbax SB-C18 column packings. Poroshell particles were synthesized as discussed in reference 9. These were bonded with SB-C18 groups in the same manner as the 1.8-µm particles in order to form the designated Poroshell 300 SB-C18 packings. All columns were prepared by conventional slurry-packing methods (1).

Results and Discussion

This overview was not intended as a vigorous theoretical or experimental treatment of the effects of particle size and type on a column plate number, pressure drop, and separation time (although these factors are closely interrelated). Such fundamental relationships are detailed in reference 1. Instead, the theme of this study was directed towards identifying some of the trade-offs between separation speed and experimental practicality. To illustrate these points, examples of various approaches for ultrafast separations were taken from recent literature describing commercially available materials and the author's laboratory.

It should be noted that for ultrafast separations, columns normally are not operated at the mobile phase flow rate (or velocity) that produces optimum column efficiency. Instead, columns are used at much higher velocities in order to reduce the separation time. The highest mobile phase velocity that can be used is determined by the pressure limitations of the column and equipment. The resulting efficiency of the column at high mobile phase velocities used for ultrafast separations is usually determined by the mass-transfer characteristics of the column packing. Therefore, column packings that show smaller losses in efficiency with increasing mobile phase velocity are usually better suited for carrying out ultrafast separations with superior resolution.

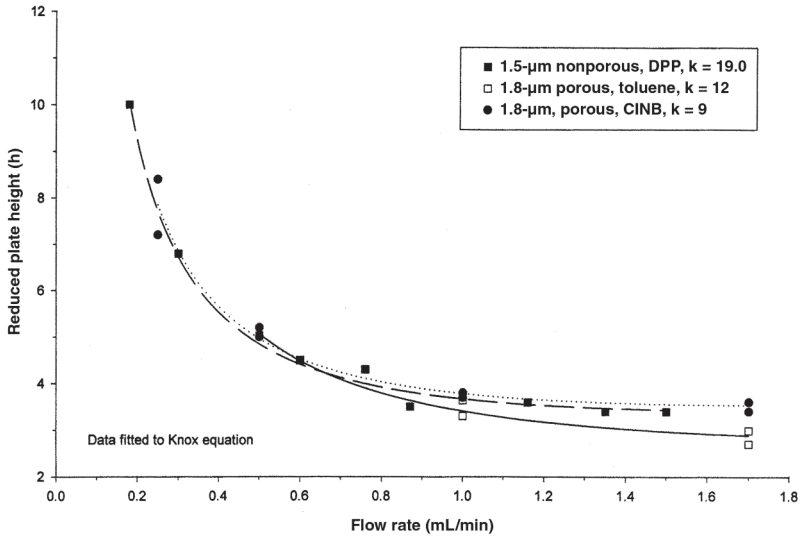

Figure 1. Reduced plate height versus flow rate plots for ultramicroparticles. Columns: 33- × 4.6-mm, 1.5-µm nonporous particles (Micra NPS C18) and 30- × 4.6-mm, 1.8-µm Zorbax SB-C18 porous particles. Mobile phase: nonporous particles, 20% acetonitrile/80% water; porous particles, 50% methanol/50% water; solutes, dipropylphthalate (DPP) for nonporous particles, 4-chloro-1-nitrobenzene (CINB) for porous particles. Data for nonporous particles were taken from reference 11.

The selection of a column with a particular particle type is based on the primary goals of the application. Very small porous particles are best suited when very fast separations of small molecules are desired (i.e., monitoring a process, determining the cut-point for a large-scale preparative separation, or in clinical and diagnostic applications). Certain combinatorial chemistry applications also appear attractive. Nonporous and superficially porous particles and monoliths appear generally most suited for the rapid separations of macromolecules. At this point, it is worthwhile to consider what are the trade-offs between separation speed and experi-

mental convenience and practicality. The following sections will attempt to put the answers to this into perspective.

Column efficiency

The performance of columns are often characterized by different forms of so-called van Deemter plots that relate the efficiency of the column to the flow rate or velocity of the mobile phase (25). The reduced plate height (column plate height/particle diameter) is often used to normalize results so that the effectiveness of the packed bed formation can be measured when columns with different particle sizes are used. Figure 1 shows such plots for three microparticular columns (one nonporous and two porous). Similar small solutes and retention (k) values were selected for this comparison in order to ensure consistency. Data for the nonporous column were taken from literature as representative of results for commercially available materials (11). The results shown for porous columns were obtained with two different columns using different solutes. Data for the columns in Figure 1 closely fit the Knox equation (25):

$$H = Au^{1/3} + B/u + Cu \qquad \text{Eq. 1}$$

where H is the plate height; u is the mobile phase velocity; and A, B, and C are constants for a particular system (25). It should be noted that in Figure 1 there is little difference in the reduced plate height minimum for the nonporous and porous columns with the mobile phase flow rate data available.

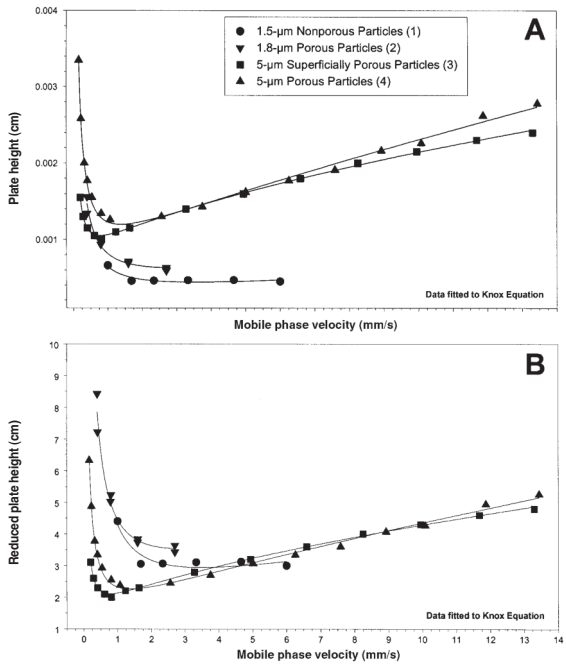

Figure 2. Plate height versus mobile phase velocity for "fast" particles. (A) 1.5-µm nonporous, Micra NPS C18: 33 × 4.6 mm; mobile phase, 60% acetonitrile/40% water; solutes, benzo[a]pyrene. (B) 1.8-µm porous particles, Zorbax SB-C18: 8-nm pores, 30 × 4.6 mm; mobile phase, 50% methanol/50% water; solute, CINB. (C) 5-µm Poroshell, 300 SB-C18: 0.25-µm shell, 30-nm pores, 75 × 2.1 mm; mobile phase, 15% methanol/85% water; solute, CINB; temperature, 24°C. (D) 5-µm porous, Zorbax SB-C18: 8-nm pores, 75 × 2.1 mm; mobile phase, 42% acetonitrile/58% water; solute, CINB; temperature, ambient. Data for the nonporous particle column were taken from reference 26.

The plots in Figure 1 also clearly show the advantage of very small particles for high speed separations (high mobile phase flow rates can be used without a significant loss in column efficiency because of their excellent kinetic properties). In these instances, the plate height minimums were not even reached for these columns at the highest flow rates (mobile phase velocities) that were practical. The downside of using high flow rates with very small particles is that very high back pressures are generated.

With columns of less than 2-µm particles, higher flow rates are required to operate at the plate height minimum compared with columns of larger particles. Lower flow rates (and mobile phase velocities) result in degraded column efficiency because of increased band broadening by solute longitudinal diffusion. Columns of ultrasmall particles can be effectively used at much higher flow rates without a significant efficiency loss, but special equipment and safety features are required for operation at the much higher pressures required (18).

Figure 2 compares column efficiency results versus mobile phase velocities for 1.5-µm nonporous particles, the 1.8-µm porous particle column of Figure 1, 5-µm SP particles with a 0.25-µm shell of 30-nm pores, and 5-µm totally porous particles (all with C18 stationary phases). Results for the nonporous particles with a C18 stationary phase were taken from literature (26). Data for the other columns were from this laboratory, and the same di-isobutyl-C18 stationary phase was used for the particles. Results for the 5-µm SP column with a small solute are given for a quantitative comparison, although the structure of this particular column packing was designed for rapidly separating macromolecules (e.g., proteins) because of its larger pore size and thin shell (9). Data in Figure 2 were obtained on systems that were not common because of requirements for different particle types. Also, some of the information was taken from existing sources and was not specially developed for this presentation. However, the results do allow semiquantitative comparisons that provide some useful insights.

Figure 2A shows the effect of mobile phase velocity on the plate height for these columns. As predicted by theory, smallest plate heights are for the smallest particles, and the plate height minimum occurs at higher mobile phase velocities for smaller particles. The plate height minimum is actually not reached for the less than 2-µm particles at the mobile phase velocities reported, these being limited by the high back pressures found at the higher velocities (greater than 400 bar for the nonporous particles).

The data in Figure 2A also show that the minimum plate heights for the totally porous and SP 5-µm particles were essentially equivalent, as predicted by theory. This feature is controlled largely by the size of the particles in the packed bed. However, the somewhat downward trend of the plate height plot at high mobile phase velocities for the SP particles is suggestive of favorable mass transfer, even for the small solute tested.

The reduced plate height plots in Figure 2B show that minimum reduced plate heights for the different particle sizes are somewhat comparable at the optimum mobile phase velocities. The smaller value for the larger particle sizes suggest that these columns were prepared with better packed beds. The optimum mobile phase velocities for the larger particles are smaller, as predicted by theory and experimentally verified in many studies (25).

The lower pressures required to operate the larger SP particles allow these materials to be used conveniently at much higher mobile phase velocities for very fast separations with a modest reduction in column efficiency. The back pressure was approximately 120 bar at the highest mobile phase velocity shown in the SP plot (approximately five times the highest mobile phase velocity for the shorter 1.8-µm porous particle column). This compares with the approximate 250-bar pressures that were required at the highest velocities for the columns of very small particles.

The closely similar plots in Figure 2 for the same-size totally porous and SP particles suggest that there is no significant advantage for the latter materials in the ultrafast separation of small molecules. The stationary-phase mass-transfer characteristics for small molecules apparently are sufficient enough to not be restrictive for the totally porous particles used in this system. However, for slower-diffusing higher molecular solutes, the SP particles with the short solute diffusion path have significantly improved efficiency at higher mobile phase velocities (26) (see also Figure 8).

The ability of columns with very small nonporous particles to separate macromolecules rapidly is shown in Figure 3. Here, seven proteins were separated by gradient elution in approximately 30 s with 1.5-µm nonporous particles coated with polystyrene (14). At a flow rate of 2.5 mL/min, this column exhibited a back pressure of 440 bar (approximately 6500 psi). The rapid separation of these macromolecules was a direct result of the excellent kinetic properties for the packed bed of these particles.

A comparison of the reduced plate height versus the mobile phase velocity plots for the SP particles with solutes of different molecular weights is shown in Figure 4. It should be noted that the experiment with insulin (MW = 5700) was performed at 60°C, which is a favorable operating environment for rapidly separating macromolecules when allowed because of the superior diffusion and mass-transfer properties. The 4-chloro-1-nitrobenzene (CINB) experiment was carried out at ambient temperature, which is typical for separating small molecules. The two sets of data cannot be quantitatively evaluated because of differences in the operating temperature and mobile phase composition.

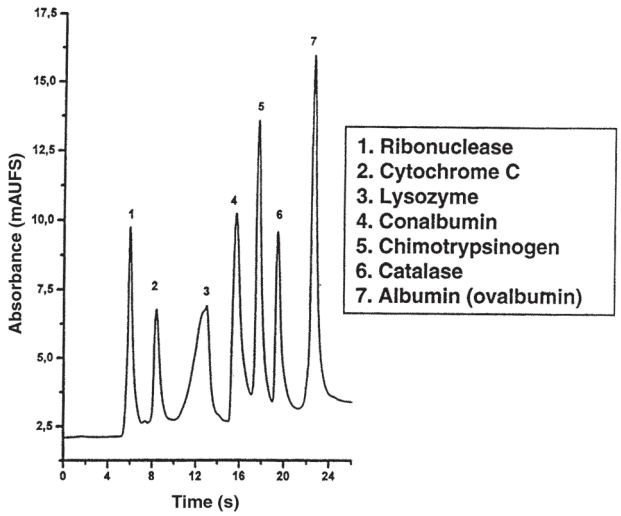

Figure 3. Rapid separation of seven proteins with a nonporous silica column. Column, 33 × 4.6 mm, 1.5-µm Micra NPS silica coated with polystyrene; mobile phase gradient, 30 to 100% of acetonitrile in water with 0.1% trifluoroacetic acid in 30 s; flow rate, 2.5 mL/min; pressure, 440 bar. Reproduced from reference 14 with permission from Elsevier Science.

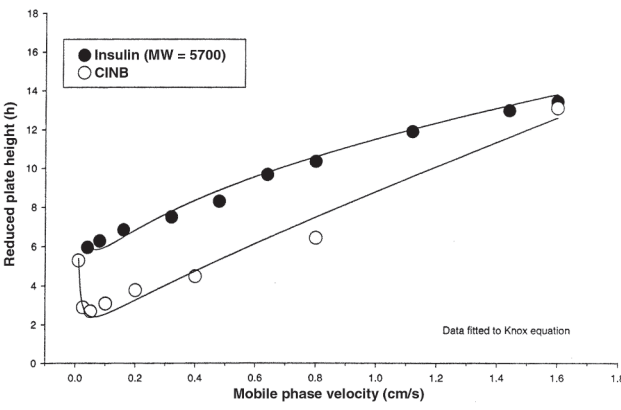

Figure 4. Reduced plate height versus mobile phase velocity plots for a Poroshell column. Column, 75 × 2.1 mm, Poroshell 300 SB-C18, 5-µm, 0.25-µm shell, 30-nm pores; mobile phase, insulin–28% acetonitrile with 0.1% trifluoroacetic acid/72% aqueous 0.1% trifluoroacetic acid; temperature, 60°C; CINB–15% methanol/85% water; temperature, 24°C. Reproduced from reference 9 with permission from Elsevier Science.

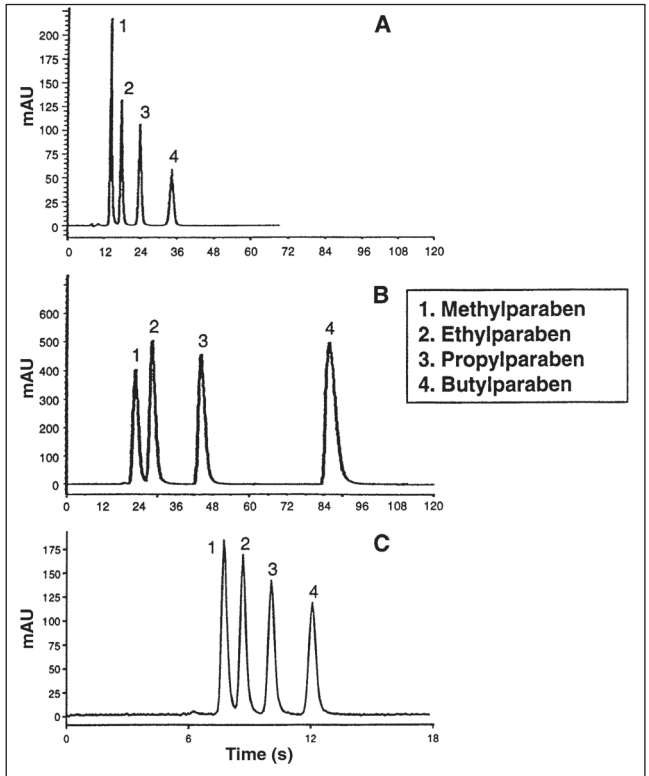

Figure 5. Rapid separations of parabens. (A) Column, 30- × 4.6-mm porous, 3.5-µm Zorbax SB-C18; mobile phase, 50% 0.1% H_3PO_4/50% acetonitrile; flow rate, 2.0 mL/min; temperature, ambient; pressure, 46 bar. (B) Column, 33- × 4.6-mm nonporous Micra 1.5-µm NPS ODS-1; mobile phase, 80% 0.1% H_3PO_4/20% acetonitrile; flow rate, 1.0 mL/min. (C) Column, 30- × 4.6-mm porous 1.8-µm Zorbax Rx-C18; mobile phase, 40% 0.1% H_3PO_4/60% acetonitrile; flow rate, 3.0 mL/min; temperature, 70°C; pressure, 198 bar; detector, UV, 254 nm. Data were taken in part from reference 27.

However, the results do allow a rough comparison of column performance. It should also be noted that the two plots in Figure 4 appear to be converging at high mobile phase velocities. This effect might be anticipated when the diffusion rate of solute molecules into the porous structure of the particles becomes less of a dominant factor in determining column efficiency.

As was previously stated, chromatographic theory predicts that the potential for faster separation speed should increase with decreasing particle size. However, this may not be a practical result (as illustrated by the chromatograms in Figure 5 for a mixture of parabens). The separation in Figure 5A with a 33-mm column of 1.5-µm nonporous particles appears inferior to the separation in Figure 5B with a 30-mm column of 3.5-µm porous particles. This is counter to theory and intuition, thus the question should be asked how this can happen. First, the assumption is that the packed beds for the two columns were appropriately optimized for efficiency, which is usually specified by a reduced plate height of 2 to 2.5 particle diameters (as for the column in the bottom plot of Figure 4). However, for the butylparaben peak, the reduced plate heights for the 1.5-µm nonporous and the 3.5-µm porous particle columns under the conditions used were 7.1 and 5.1, respectively, which suggests that the latter column with larger particles had a superior packed bed. This difference should not be surprising, because it is well-known that it becomes increasingly more difficult to pack efficient column beds as the particle size is decreased.

Secondly, the assumption is that the separations in Figures 5A and 5B have been optimized for speed, thus maintaining approximately the same resolution. However, it becomes difficult to make direct comparisons of different particle types, because experimental requirements are different for producing optimum separations. For example, in order to approximately maintain the same k values, the nonporous particles in Figure 5B require a lower concentration of organic modifier. It should also be noted that the flow rate used for the separation with porous particles was twice that of the nonporous separation. Therefore, in order to make more quantitative separation comparisons, the need for different operating parameters must be taken into account.

With 1.8-µm totally porous particles of the same type as the 3.5-µm particles used in Figure 5B, it was feasible to produce an even faster separation of the neutral parabens (as illustrated in Figure 5C). This 3.0-cm column of 1.8-µm particles was operated at 3.0 mL/min (approximately 13.5 mm/s) and 70°C in order to produce an isocratic separation in less than 14 s with excellent resolution. This very fast separation was facilitated by using a higher column temperature, which reduced the viscosity of the mobile phase (allowing faster flow rates) and improved diffusion of the solutes. Ultramicroparticles can also be used for the very rapid separations of ionizable compounds (as shown in Figure 6 for a mixture of drugs and related compounds). This 25-s isocratic separation of seven components to better than baseline resolution was accomplished at the cost of a higher flow rate (3.0 mL/min) with an accompanying back pressure of 232 bar. The use of the highly stable sterically protected di-isobutyl-C18 stationary phase for this column permitted higher operating temperatures, which improved mass transfer and allowed a faster separation.

Very fast separations of macromolecules can also be performed with columns of properly designed monoliths (as shown in Figure 7). Seven proteins were separated by gradient elution in approximately 20 s on this monolith column of an organic polymer. For this separation, a flow rate of 10 mL/min was required for the separation speed (7).

As previously indicated, the wide-pore SP particles of this study were specifically developed for the fast separation of macromolecules (8,9). The stationary phase located in the thin porous shell of these particles was rapidly accessed for interaction, thus favoring large molecules with poor diffusional properties. This particle structure may be less favorable for rapidly separating small molecules than very small totally porous particles (8).

With SP particles, compounds with slow diffusion rates have short distances for interaction, which leads to the capability of the thin porous shells to produce very fast separations of large molecules at high mobile phase velocities. An illustration of this is shown in Figure 8 in which the gradient separation of eight proteins is performed in approximately 50 s. It should be noted that the good efficiency and high peak capacity of this column could allow the separation of even more components in the same time span. A specific advantage of larger SP particles over ultramicroparticles for very fast separations is that longer columns can be used with lower pressures in order to obtain the high peak capacity needed for separating more complex mixtures.

Therefore, ultramicroparticle, monolith, and SP columns are clearly capable of great separation speed. However, it is important to consider what are the experimental compromises that must be met to get this level of performance. These factors are discussed in the sections that follow.

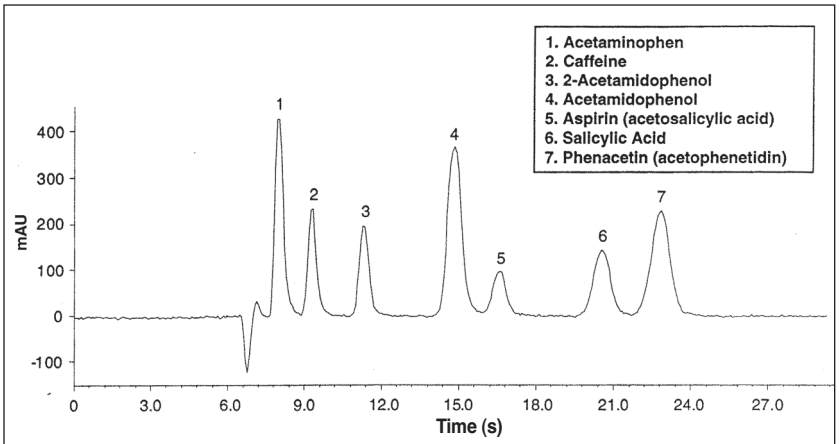

Figure 6. Fast separation of analgesics. Column, 30- × 4.6-mm porous, 1.8-µm Zorbax SB-C18; mobile phase, 25% acetonitrile/75% water (both with 1% formic acid); flow rate, 3.0 mL/min; temperature, 70°C; pressure, 232 bar; detector, UV, 220 nm.

Detection

More efficient columns create sharper bands of lower volume. Therefore, columns that elute peaks in the smallest volume allow the lowest detection limit because the solute mass is concentrated in a smaller volume. For the same column size and separation parameters, detection sensitivity is in this case a direct function of column efficiency. Column efficiency involves not only

particle size but also how well the column is packed (as indicated in the previous discussion on efficiency).

Detection sensitivity can also be a function of the detector response and the data-handling system used. Because of the rapid elution of peaks such as those in Figures 4 to 6, the rate of collecting data should be sufficiently fast in order to ensure accurate digitization of the peaks of interest (28). It should be noted that in Figure 9 a commonly used detector response time of 2 s badly degraded this separation with a 15- × 4.6-mm column of 3.5-µm particles. In this case, accurate peak configuration occurred only when a response time of 0.5 s or less was used. Similar or smaller volume columns of even smaller particles may require the fastest available detector response time. The same reasoning applies to the data-sampling rate for the data-handling system used. For very fast separations of the type shown in Figure 5C, data-sampling rates of 10 points/s or greater are often required. Figure 10 shows the enlarged lysozyme peak for a separation using SP particles similar to that in Figure 8. Distinct data collection points were defined showing that the detector/data sampling rate used was barely able to adequately define the peak shape with 12 data points or approximately 13 data points per second.

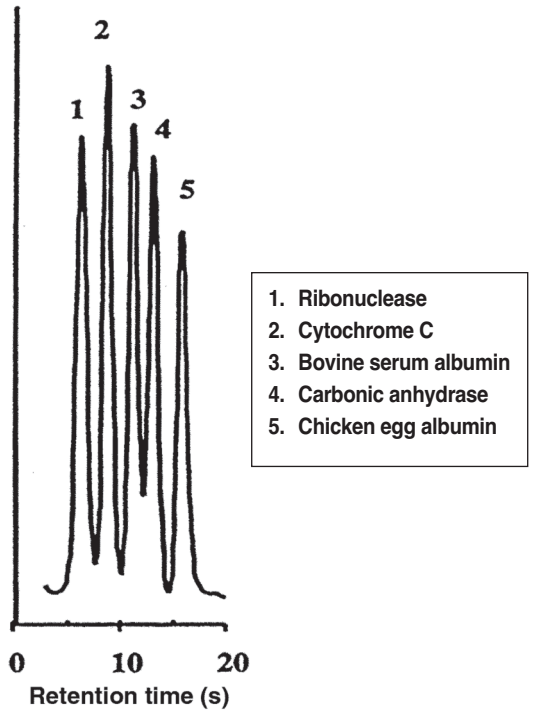

Figure 7. Rapid reversed-phase separation of proteins with a monolith column. Column, 50- × 4.6-mm Monolith A; mobile phase gradient, 42 to 90% acetonitrile in water with 0.15% trifluoroacetic acid in 0.35 min; flow rate, 10 mL/min. Reproduced from reference 7 with permission from Elsevier Science.

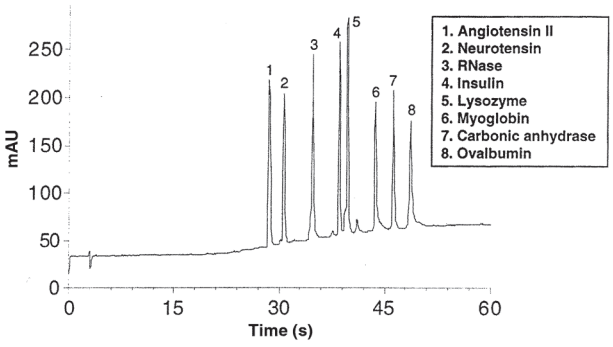

Figure 8. Fast separation of peptides and proteins with a column of superficially porous particles. Column, 75- × 2.1-mm 5-µm Poroshell 300 SB-C18 (0.25-µm porous shell); mobile phase gradient, (A) 0.1% trifluoroacetic acid, (B) 0.07% trifluoroacetic acid in acetonitrile, 5–100% B in 1.0 min; flow rate, 3.0 mL/min; temperature, 70°C; pressure, 260 bar; detector: UV, 215 nm.

Sample recovery

When compared with totally porous particles with the same stationary phase, lower surface-area nonporous ion-exchange particles produce higher yields of certain proteins (29). Whether this trend is continued with all reversed-phase packings is not yet fully documented. However, if recovery of the solute type is influenced by the amount of stationary phase that is accessible for irreversible binding and thus loss of analyte, one might expect that the nonporous particles might have more favorable properties for the recovery of very low amounts of solute. Compared with totally porous particles, columns of SP particles may also have an advantage in terms of recovery yield because of the lower surface area.

Sample isolations

Because of the much higher surface area of porous particles, columns of these materials are more useful when larger quantities of a purified solute must be isolated. For particles of the types described for Figure 1, 10 to 100-fold more sample can be loaded onto the porous column compared with the nonporous column before severe degradation of the resolution occurs. A 1.5-µm porous silica particle closely similar to that in Figure 5B with a C4 stationary phase showed a solute loading of 20 µg/g of packing before a 10% decrease in the k factor was seen for insulin (5). Higher loadings might be expected for a C18 stationary phase, especially for small molecules.

Short columns of porous ultramicroparticulates are useful to rapidly isolate purified materials for chemical characterizations and other tests requiring microgram to submilligram quantities. The yield of purified material per run is usually based on the total surface area available within the column. Columns of nonporous particles can be used to isolate the much smaller amounts needed for mass spectrometry (MS) and other such sensitive methods.

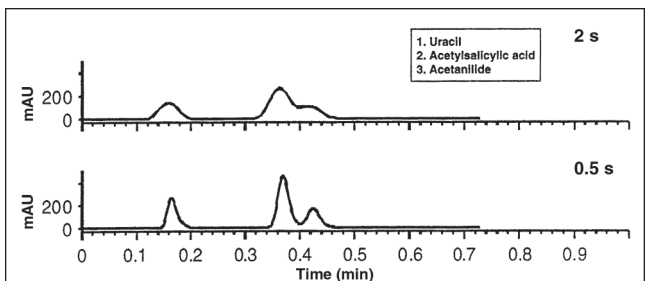

Figure 9. Effect of detector response time with fast separations. Column, 15- × 4.6-mm 3.5-µm Zorbax SB-C18; instrument, Agilent Technologies 1100; mobile phase, 32% acetonitrile/68% 1% formic acid; flow rate, 1.0 mL/min; temperature: 30°C; injection volume, 5 µL. Time constants are shown in seconds. Data were taken from reference 28.

Because of the higher surface areas afforded by the porous shells, SP particles would be expected to allow larger amounts of purified fractions to be isolated compared with nonporous particles, but less than totally porous particles (9). Polymer-based monolith columns have exhibited reasonably high sample loading capacities (7).

Equipment requirements

The effective use of both nonporous or porous ultramicroparticulates requires well-designed instruments that minimize extra-column band broadening (4,11,14,30). The reason for this is that columns of these particles produce very sharp low-volume peaks (as mentioned in the above discussions of detection and separation speed). The result of extra-column band effects is the broadening of the peaks from the analytical column (1). Equipment not designed for low-volume high-speed columns will provide inferior isocratic separations, not unlike the degraded peaks in Figure 9A for slow-responding detectors. Proper equipment design is also of special importance for gradient elution, because large holdup volumes will contribute to the gradient delay and gradient rounding that affect separation speed and reproducibility. Devices that poorly form very rapid gradients also contribute to separation irreproducibility.

Peaks formed by ultramicroparticles are extremely low in volume and sharp; therefore, equipment demands for columns of these particles are very high. Because of the very small peak volumes associated with ultramicroparticles, columns of these materials are often made with wider internal column diameters in order to produce larger peak volumes that are more compatible with conventional HPLC equipment. For such ultramicroparticles, column dimensions of 30 × 4.6 mm are typical. Longer columns of these materials generally produce column back pressures that are too high for routine operations. The larger particles used in columns of SP particles produce larger-volume peaks; therefore, a larger range of column dimensions can be used. In order to increase solute mass sensitivity and reduce mobile phase volumes, column dimensions of 50 to 100 × 2.1 mm appear optimum (but not limiting) for SP columns. For the fast separation of proteins and DNA fragments, 75- × 2.1-mm columns of SP appear to be useful (9). For these column dimensions, HPLC equipment with microdetector cells and injectors and conventional data-handling systems can be satisfactorily used without other modifications.

Column inlet effects

Although columns of 3.5- and 5-µm particles commonly use end-fittings with 2-µm porosity frits that are not easily plugged by particulates, one special disadvantage of columns with very small particles is that they use easily plugged 0.5-µm frits. This means that most samples must be carefully pretreated by filtration, centrifugation, or other means before separation. The addition of an in-line 0.5-µm filter between the sample valve and the column inlet is useful for minimizing column pluggage problems. However, such a filter should only be used when it is verified that it does not create extra-column band broadening that degrades the separation of interest. Because conventional 2-µm porosity frits are used on columns of larger SP particles, traditional sample pretreatment and guard column techniques are generally adequate for long-term operation.

Mobile phase consumption

The faster separations with ultramicroparticles reduce the quantity of mobile phase per separation compared with separations of the same efficiency with columns of larger particles. The reason for this is that the shorter lengths used for columns of ultramicroparticles result in shorter retention times.

Because of the lower stationary phase concentration on nonporous and SP packings, retention is less than for porous packings when using the same operating conditions. Therefore, a mobile phase with a lower concentration of organic modifier (higher water content) is typically needed for separations with the same k values for these type of packings compared with totally porous particles. Although this situation represents a savings in organic solvents, it can be a problem for samples with very limited solubility in water, especially for preparing sample solutions for injection. Separation reproducibility for certain biomacromolecules can be favored by a higher concentration of organic mobile phases, which tend to more completely denature the molecules for better separation reproducibility. Higher organic composition in the mobile phase can be of significant value when maintaining the solubility of highly hydrophobic (poorly water soluble) solutes during a reversed-phase separation such as in the separation of amyloids from brain tissues (31). In such cases, the use of more retentive columns with totally porous particles can be an advantage.

Separation ruggedness

The ruggedness and repeatability of HPLC separations is dependant on several factors, including column stability and lifetime and the influence of changes in separation parameters. Insufficient systematic data on the long-term stability of monolith

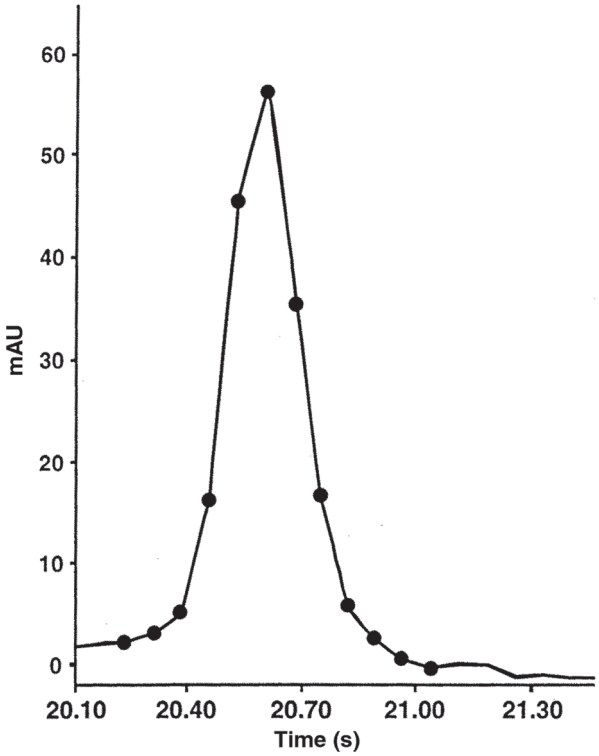

Figure 10. Detector data point settings for fast separations. Conditions were the same as those in Figure 8; UV detector settings, 13 points/s.

columns and columns with ultramicroparticles and SP particles are available, thus this issue still has to be fully documented. One form of separation instability is because of packing degradation by chemical attack on the bonded stationary phase or the silica support. At a low pH level, the bonded silane stationary phase can be hydrolyzed and lost from the silica particle; high pH mobile phases slowly dissolve the silica and ultimately result in bed collapse (32). More than 3000 column volumes of a pH 10 ammonium acetate mobile phase has been reported for one nonporous packing before degradation was noted. Also, no effect at pH 2 was reported, but use or test conditions were not described (33). Under optimum conditions, 5-µm porous silica columns with optimum stationary phases showed little change after approximately 30,000 column volumes of mobile phase underwent passage at both pH 0.9 and 90°C (34) and pH 11 and 40°C (32). Data on similar 2-µm porous particles were not yet available.

Unfortunately, no head-to-head tests comparing nonporous, SP, and porous columns under the same conditions at low or high pH have been reported. Unless the same or closely similar conditions are used for the two packings, no valid conclusions regarding the effects at low or high pH can be drawn. However, it seems clear that because of the smaller amount of stationary phase on the low surface-area nonporous particle, any change in this stationary phase (either by loss or contamination) would cause more change in chromatographic characteristics than for the porous particles with a higher surface area and larger amounts of stationary phase. The effect for SP particles should be intermediate because of the intermediate surface area. More studies on the stability effects in low, intermediate, and higher pH mobile phases are needed.

Another aspect of separation ruggedness is the ability of the column to resist changes in solute retention and resolution with changes in operational parameters. Figure 11 shows the effect of mobile phase composition on the retention of solutes for the two column types (nonporous and porous). For a drug intermediate in Figure 9, the plot of log k versus the organic modifier concentration (%B) for the nonporous column was extremely steep. A small change in %methanol caused a large change in the retention time. Less change in retention with change in %B was seen for the porous column, as would be predicted by the much larger phase ratio (larger amount of available stationary phase) of this system. There is also a general tendency for the slope of such log k versus %organic modifier plots to increase with lower values of %B or solute retention (35).

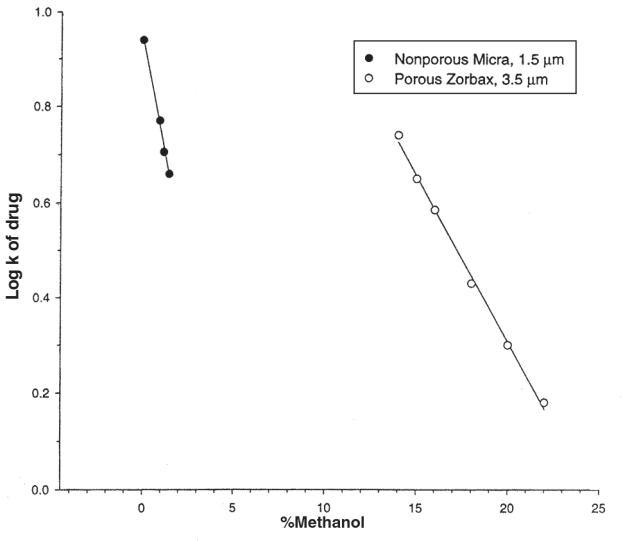

Figure 11. Effect of mobile phase composition on nonporous and porous particles. Columns, 33 × 4.6 mm, 1.5-µm Micra NPS C18; 150- × 4.6-mm Zorbax SB-C18; flow rates, 1.0 mL/min. Data were taken from reference 11.

Therefore, separation ruggedness is much less favored for columns of nonporous particles because of the great sensitivity of retention to even small changes in %B and other operating parameters. It should also be noted in Figure 11 the large difference in the level of organic needed to obtain the same retention for this solute. Almost one order of magnitude lower of organic concentration was required for the same retention with the nonporous column, which shows the influence of the smaller amount of available stationary phase. Because of the intermediate surface area of SP particles, the effect of the %organic modifier on retention is also expected to be somewhat intermediate.

The results in Figure 11 indicate that columns of porous particles are less susceptible to change with changing separation parameters largely because of the higher phase ratio. The effect of temperature and pH changes for ionizable solutes would be expected to be similar to that for %organic changes (36,37). Therefore, separation ruggedness is favored for porous particles with higher surface areas and higher amounts of stationary phase.

Method ruggedness might also be influenced by the strength of very small particles that must be packed into column beds at high pressure. Nonporous particles are mechanically stronger than porous particles because of their denser structure. However, most of the 5- and 3-µm porous silica microspheres used today have adequate strength for HPLC requirements. One would anticipate that 2-µm porous particles would have similar strength, but definitive data on this subject are not available. No structural problems were encountered in previous studies with less than 2-µm porous particles (5), and no difficulties were noted in similar published accounts (20) or the separations associated with the column of Figures 4C and 5. SP particles are extremely strong and present no problems in the high-pressure packing of columns and use at high pressures (8,9).

Separation reproducibility and method ruggedness is also dependent on the ability to reproduce the separating bed from column to column. Many studies have demonstrated that discrete spherical particles of ≥ 3.5 µm can be used to produce columns with highly reproducible chromatographic characteristics. Therefore, the reproducibility and ruggedness of such systems has been widely documented. However, data documenting the reproducibility of routinely preparing columns of ≤ 2-µm particles and monolith columns have not been disclosed, as far as the author is aware.

Retention of lightly held solutes

Because of the higher surface area and phase ratio, porous particles inherently have greater retention for solutes with the same mobile phase (see Figure 11). Consequently, when greater retention for lightly held solutes is needed, columns of porous particles with higher surface areas will always be favored. This effect can be especially important when separating more hydrophilic polar compounds.

Use with hyphenated methods

Because of the higher speed and lower volume peaks associated with less than 2-µm particles, these materials have advantages over larger particles for LC–MS and other fast, highly sensitive hyphenated methods that require very small samples for operation. However, there would appear to be no special advantage for either nonporous or porous particles in these instances. If higher peak capacity is required for separations, columns of SP particles may have an advantage over ultramicroparticles. In this case, the lower back pressures of larger SP particles would allow for the longer columns needed to produce the required higher peak capacities. Longer monolith columns can also be used to develop higher peak capacities because of the lower back pressures of these units. For hyphenated methods that prefer mobile phases with less water, porous particles may be advantageous. For mass-sensitive devices such as radioactive detectors, fast peaks are a detriment, thus columns with slower broader peaks afforded by larger particle sizes would be preferred.

Column back pressure

The back pressure of similarly packed beds is the inverse square function of the particle diameter and is usually not a function of porosity. Therefore, the higher pressures of columns with very small particles require that shorter columns of these materials be used. For columns of 2-µm particles, column lengths of approximately 30 mm are near maximum because of the pressures required for efficient operation. A column of 5-µm SP particles can be much longer because of the larger particle sizes used. Studies with 150-mm SP columns of the particles have been successfully completed without problems (38). As previously indicated, monolith columns inherently generate lower back pressures compared with typical packed beds. This feature allows the use of very high mobile phase flow rates for very fast separations, as illustrated in Figure 7.

Column regeneration

The ability to regenerate the column after a solvent change or gradient run has a large effect on the total time required for analysis. For instances such as this (although no comparative studies have been published) nonporous and SP particles would appear to have an advantage because of their lower surface areas with corresponding lower amounts of stationary phase. Very fast regeneration has been claimed for nonporous columns. This effect would appear to be valid in view of the low surface area and stationary phase concentration for these materials. Based on the surface areas, one would expect that columns of SP particles would show regeneration effects more similar to nonporous rather than totally porous particles. Regeneration of monolith columns should be relatively convenient because of the low back pressures and higher flow rates that can be used.

Conclusion

This overview compares the practical aspects of nonporous, porous ultramicroparticles (≤ 2 µm), monoliths, and superficially porous particles (approximately 5 µm) for conducting very fast separations. Columns of each of these particle types have specific advantages and disadvantages for particular uses. The comparative properties of particle types for the different aspects of separations are summarized in Table I.

When optimized, columns of all of these materials are potentially capable of separation times of less than 1 min for many sample types. Such very high separation speeds favor high-throughput applications such as those required in combinatorial chemistry and for monitoring rapidly changing systems such as process applications. Columns of totally porous ultramicroparticles generally are best suited for separating small molecules. Nonporous ultramicroparticles and superficially porous particles have the greatest strengths for rapidly separating macromolecules that have poor diffusional characteristics. With present technology, columns of ultramicroparticles are less convenient for routine applications because of extra-column effects and potential problems with guarding the columns from pluggage. Columns of the larger superficially porous particles can be used with conventional equipment and techniques, but widespread applications of these columns have not yet been developed. Applications with monolith columns are sparse, but this approach also appears to have potential for rapidly separating macromolecules.

Table I. Relative Advantage of Particle Types Versus Widely Used 5-µm Porous Particles

Property related to fast separations	Particle characteristics* Porous 3.5 µm	Porous 2 µm	Nonporous (1.5 µm)	Superficially porous (5 µm, 0.25-µm shell)†	Monoliths
Separation speed	♦	♦♦	♦♦	♦♦	♦♦
Detection	♦	♦♦	♦♦	♦	0
Sample recovery	0	0	♦	♦♦	0
Preparative isolations	0	–	––	–	0
Equipment requirements	0	–	––	0	0
Mobile phase volume used	♦	♦♦	♦♦	0	–
Reproducibility, ruggedness	0	–	––	0	–
Retention of lightly held solutes	0	––	–	0	
Use with hyphenated methods	0	♦	♦	0	0
Back pressure	–	––	––	0	♦♦
Fast column regeneration	0	0	♦	0	♦
Typical column dimension (mm)	150 × 4.6	30 × 4.6	33 × 4.6	75 × 2.1	50 × 4.6

* Specific particle sizes are nominal, but generally representative: narrow pore size (i.e., 8–10 nm) for separating small molecules assumed.
† This particular material was specifically designed with 30-nm pores for separating macromolecules. Superior separation speed is expected only for macromolecules and not for small molecules, as indicated by the data in Figure 2.
♦, Advantage.
♦♦, Strong advantage.
0, No advantage.
–, Disadvantage.
––, Strong disadvantage.

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