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High velocity reversed-phase chromatography of proteins and peptides: use of conventional C18, 300 Å, 15 μm particles

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Abstract

Experiments were conducted to study the effects of mobile phase velocity on the reversed-phase chromatography of peptides and proteins using a mono-modal pore size (300 Å) C18 spherical silica packing. This material was packed into several 5 × 0.46 cm columns for gradient elution studies using ribonuclease A, insulin, lysozyme and myoglobin. Baseline separation of these proteins was achieved within 90 seconds. Using a two-minute linear gradient from 15 to 65% acetonitrile (in 0.1% trifluoroacetic acid), resolution improved with velocity. Enhanced performance was attributed to the concurrent increase in gradient volume with higher mobile phase velocity.

The frontal adsorption capacity of lysozyme was 25 and 23 mg/ml at 220 and 3600 cm/h on the 300 Å packing material. These values are equivalent (within an experimental error of ± 2 mg/ml) clearly demonstrating that lysozyme (M_r 14 300) fully permeates the 300 Å pores during operation at high mobile phase velocity. Comparison of protein diffusion velocity with the distances involved in pore penetration substantiates the feasibility of this observation. Loading studies were conducted at both 360 and 3600 cm/h using the protein test mixture. The resulting chromatograms were very similar indicating that, under certain circumstances, separations can be run on conventional particles at velocities 5 to 10 times greater than currently practised. The preparative implications are discussed.

1. Introduction

From its inception, high-performance liquid chromatography has been principally concerned with separation speed. Indeed, one of the earlier designations for the technique was high speed liquid chromatography (HSLC). The driving force behind the quest for speed in analytical liquid chromatography has ostensibly been the need to perform more and more analyses per unit time, especially (but not exclusively) in the pharmaceutical industry. Although speed is also of importance in preparative chromatography, it does not seem to be as significant. Whereas for analysis the objective is to resolve and quantitate all components of a mixture, as quickly as possible (on the analytical scale, the most costly component is time), the goal in preparative chromatography is to isolate the maximum amount of pure material (yield) as economically as possible within a given time constraint. There is a classic interrelationship between load, yield and speed in preparative LC as indicated in Fig. 1.

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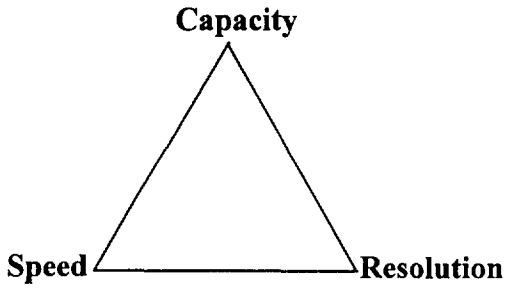

Fig. 1. The Preparative Triangle.

The utility of speed in preparative chromatography cannot be represented by separation time alone, but by the amount of purified material isolated per unit time (production rate) or alternatively, by the cost of producing the desired quantity of product in the required time. If accelerating the process decreases the production costs, then it can be justified. If, however, the acceleration is achieved at the expense of yield, the net value diminishes. This is especially important in the separation of biomolecules which have a potential value in great excess of the other associated purification costs.

In recent years, there have been several publications which discuss the advantages of speed for the preparative chromatography of proteins and peptides [1–5]. Although much of this work involved the use of macro-reticulated packings with bi-modal pore distributions for the ion exchange purification of proteins, results have also been presented for the preparative reversed-phase chromatography of peptides and small proteins [6,7]. These papers implied that the test mixtures chosen could be separated at velocities 5 to 10 times greater than those used conventionally on packings with mono-modal intraparticle pore size distributions.

The application of reversed-phase chromatography for the analysis and isolation of small proteins and peptides has been well established [8–10]. In fact, the technique is so powerful that it is often the only method capable of separating polypeptide chains which differ only in oxidation state or have undergone deamination. In general, silica-based packings with pore diameters of around 150 to 300 Å have been widely used for these separations, with great success. The wide pores are thought to be necessary for the facile access of larger biomolecules, although some data exists [11] which suggest that preparative separations could be carried out on even smaller pore diameter packings to take advantage of the higher surface area. Although there are numerous publications on the conventional use of these silica packings (i.e. at flow velocities of around 300–1000 cm/h), none have investigated the limits of speed.

The work presented here addresses the issue of speed as it applies to the preparative reversed-phase chromatography of small proteins and peptides using a spherical, 300 Å, C18 silica. The 300 Å pore diameter is commonly employed in polypeptide chromatography and silica gels of this type have surface areas which are acceptable for preparative applications [11]. Material of 15 μm particle diameter was chosen, since it offered a good compromise between efficiency and interstitial permeability. A sample mixture consisting of insulin (M_r 6000), ribonuclease A (M_r 13 700), lysozyme (M_r 14 300) and myoglobin (M_r 17 200) was used. These biomolecules have diffusion coefficients in the order of 1×10^{-6} cm^2/s [12] and facile access to the intraparticle surface area [11]. As such, chromatography at higher velocities (>500 cm/h) appeared plausible.

2. Experimental

2.1. Equipment

A Waters Millennium 2010 HPLC system (Waters Associates, Hartford, CT, USA) consisting of two 510 pumps and a photodiode array detector was used. System operation and data acquisition were performed by Millennium software.

2.2. (Bio)chemicals and solvents

Phe-Gly-Phe-Gly, ribonuclease A (bovine), insulin (bovine), lysozyme (egg white) and myoglobin (equine) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The ace-

tonitrile (ACN) and trifluoroacetic acid (TFA) were HPLC grade and purchased from a local distributor. Bulk Poros 20 R-2 was purchased from Perceptive Biosystems (Cambridge, MA, USA).

2.3. Physical testing

Mercury porosimetry and Malvern particle size analyses (by laser light scattering) were conducted by the analytical services department of the W.R. Grace Washington Research Center (Columbia, MD, USA).

2.4. Chromatography

The C18-300Å-15 μm spherical silica was prepared from Amicon 300 Å, 15 μm spherical silica gel by standard silanization techniques. The C18-250Å-20 μm granular packing was prepared from an Amicon 250 Å, 20 μm irregular silica gel. Approximately 600 mg of each C18 silica were required to fill a 5 × 0.46 cm HPLC column. Columns were packed by a downward slurry protocol using methanol as push solvent. A stock solution of 1 mg/ml each of insulin, ribonuclease A, lysozyme and myoglobin was prepared in 15% acetonitrile, in 0.1% aqueous TFA. Mass loads were varied by changing the injection volume. Mobile phases for the gradients were: Solvent A, 10% acetonitrile in 0.1% aqueous TFA; Solvent B, 70% acetonitrile also in 0.1% aqueous TFA. The instrument was then used to adjust the gradient time and mobile phase acetonitrile composition, accordingly.

3. Results and discussion

3.1. Physical characterization

Several physical analyses were conducted to characterize the C18-300Å-15 μm spherical silica gel. Microscopic examination at 400 × showed that more than 95% of the particles were spherical, based upon manual counting of a 500-particle field. Furthermore, Malvern particle size analysis (by laser light scattering) showed the average particle diameter to be 15 μm with a d_{90}/d_{10} ratio of 2.5. These results, along with other analyses, are listed in Table 1.

Single point BET (nitrogen adsorption) analysis has been used widely for the determination of surface area. This information, in addition to pore volume data, can be used to calculate pore diameter [13]. An alternative method of determining pore diameter is by mercury porosimetry (or Hg intrusion analysis), where mercury is forced around and into the particles under increasingly higher pressure [14]. The resulting (semi-log) pore volume versus pore diameter plot can then be related to the material's interstitial volume (macropores), intraparticle pore volume (micropores) and pore size distribution. It is the preferred technique for the analysis of wider pore particles which are out of the nitrogen sorption measurement range [14].

Fig. 2a illustrates the porosimetry results for the (unbonded) 15 μm spherical silica used in this study. As expected, the average intraparticle diameter was 300 Å with a reasonably tight distribution. The average interstitial space was about 5 μm. These results are typical for a "conventional" (i.e. mono-modal pore distribution) silica-based HPLC packing material. In contrast, Fig. 2b shows the pore distribution for Poros 20 R-2 which is a polymeric, reversed-phase, "perfusive" type particle. Although, in our hands, the bi-modal pore distribution is not evident (as reported in previous literature [1–5]), the mean pore diameter (1800 Å) was substantially larger than that of the silica gel (300 Å).

Table 1
Physical properties of C18-300Å-15 μm spherical silica

Shape	>95% spherical
Nominal particle diameter	15 μm
d_{90}/d_{10}	2.5
Pore volume	0.8 ml/g
Surface area	85 m^2/g
Nominal pore diameter	300 Å
Carbon content	7% (w/w)
Packing density	0.48 g/ml

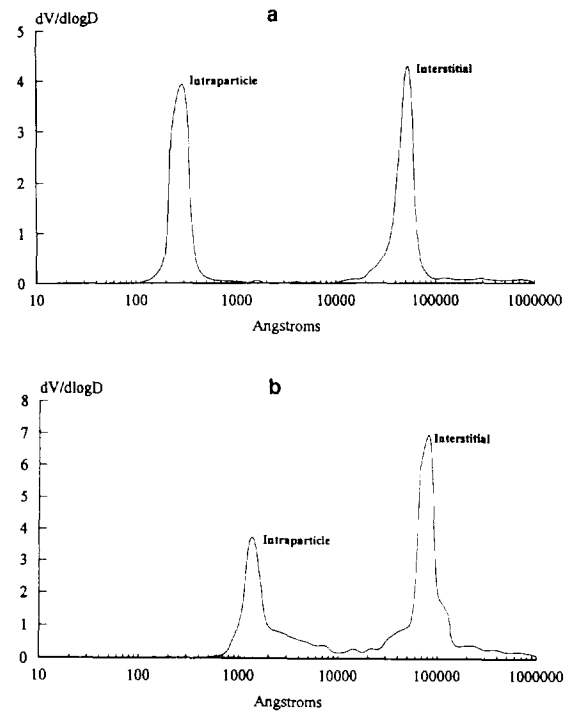

Fig. 2. Mercury porosimetry analyses conducted on the 300 Å-15 μm bare silica (a) and Poros 20 R-2 (b). The data has been plotted in a semi-log format as the change in pore volume/log of the pore diameter (dV/dlog D) vs. pore diameter.

3.2. Chromatographic characterization

Several 5 × 0.46 cm columns with C18-300Å-15 μm were packed for the study. A representative graph plotting HETP vs. flow velocity for both toluene and the tetrapeptide Phe-Gly-Phe-Gly is shown in Fig. 3. The mobile phases used were 50% aqueous acetonitrile for toluene and 18% acetonitrile in aqueous 0.1% TFA for the peptide. HETP values where measured under retained conditions with a capacity factor of around 2. Although HETP values derived from non-retained solutes have been used to characterize packings for high speed operation [1], the physical meaning of these data has been unclear.

The number of plates in the 5 cm column ranged from 500 to 310 for toluene and from 210 to 150 for the tetrapeptide over the range of linear velocities from 500 to 3600 cm/h. The

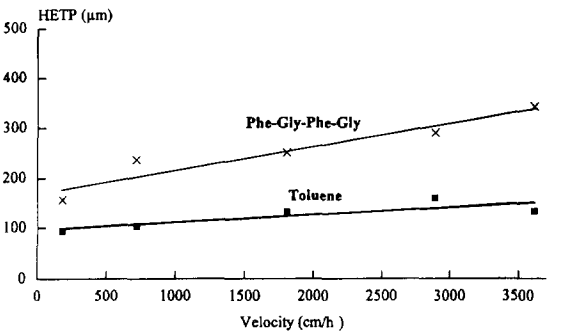

Fig. 3. HETP vs. velocity curves under retained conditions generated for both toluene and Phe-Gly-Phe-Gly on a 5 × 0.46 cm column containing C18-300Å-15 μm. Analytical injections (10 μl of a 1 mg/ml sample) were made at the indicated velocities. HETP's were calculated per the half-height equation. Mobile phases of ACN–H_2O (50:50, v/v) and (18:82, v/v) (both with 0.1% TFA) were used for toluene and Phe-Gly-Phe-Gly, respectively. The capacity factor for both solutes was ca. 2 and detection was by absorbance at 254 or 220 nm.

experimental plots were fitted to the Knox equation using a "B" term of 2 and reasonable values for the diffusion coefficients. The Knox "A" coefficient value was 2.5 indicating that the column was not ideally packed. However, the performance was sufficient for the purposes of this study.

3.3. Influence of velocity on gradient elution

High velocity reversed-phase gradient elution chromatography of proteins was conducted on C18-300Å-15 μm particles achieving separation times under 90 s (see Fig. 4). This separation was performed using a flow rate of 10 ml/min (superficial velocity 3600 cm/h) with a 2-min gradient. Given the current thinking that proteins diffuse too slowly for rapid separations on conventional (or mono-modal) 15 μm particles, the data were somewhat unexpected. One possible hypothesis may reside in the fact that the reversed-phase separation of these four proteins was relatively easy, since the column has only a few hundred theoretical plates. Difficult separations would (by definition) require more plates and therefore would not be amenable to operation at higher

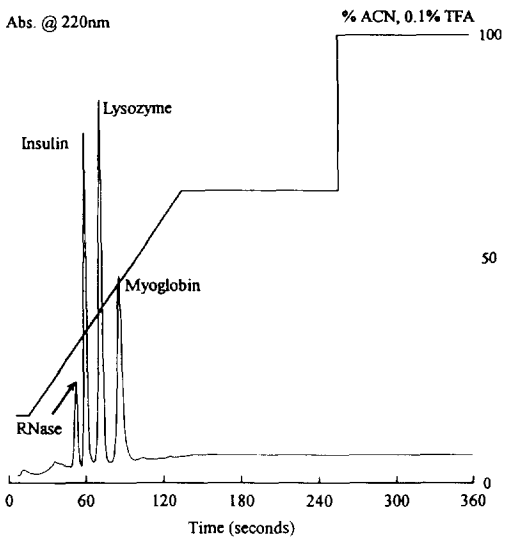

Fig. 4. High velocity reversed-phase chromatography of proteins. The separation was conducted on a 5 × 0.46 cm column of Amicon C18-300Å-15 μm spherical silica. The injection was 20 μl of a 1 mg/ml sample (each) of ribonuclease A, insulin, lysozyme and myoglobin. Proteins were eluted during a 2-min linear gradient at 3600 cm/h (10 ml/min) from 15% ACN to 65% ACN (with 0.1% TFA). Solutes were detected by absorbance at 220 nm.

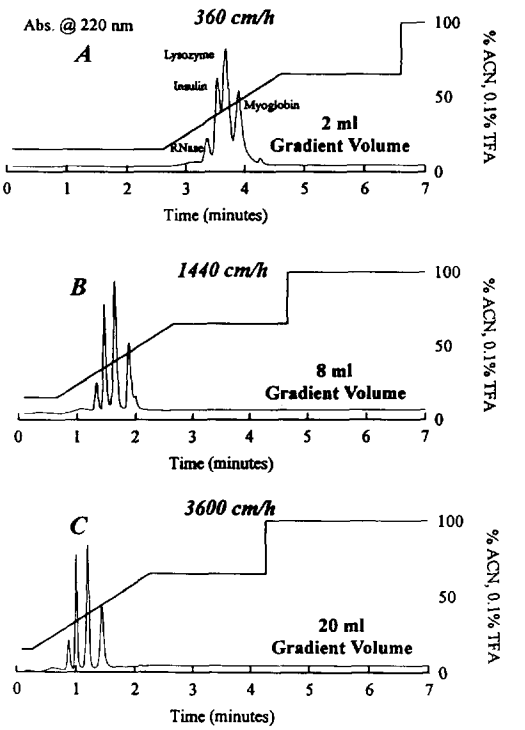

Fig. 5. Influence of velocity on resolution using a C18-300 Å-15 μm spherical silica gel. Chromatograms A–C illustrate the gradient elution profiles of ribonuclease A, insulin, lysozyme and myoglobin, as a function of linear velocity using a 5 × 0.46 cm column containing C18-300Å-15 μm spherical silica. The volumetric flow rates corresponding to 360, 1440 and 3600 cm/h were 1, 4 and 10 ml/min, respectively. A 20-μl injection was made of a solution containing 1 mg/ml of each protein. A 2-min linear gradient from 15% ACN to 65% ACN (with 0.1% TFA) was maintained and the gradient volumes are indicated in each figure. Solutes were detected by absorbance at 220 nm.

mobile phase velocity. Obviously, difficult separations tend to be the norm.

The elution of adjacent protein bands from the C18 surface differed by about 3 to 5% acetonitrile. Desorption appears to be relatively rapid. During the course of gradient elution, the proteins will rapidly desorb when the critical concentration of acetonitrile is achieved. As such, improved resolution can be attained by increasing the mobile phase velocity which concurrently increases the gradient volume (at constant gradient time). In effect, more volume is interjected between the peaks, thus improving the apparent resolution. It should be noted, therefore, that comparisons of gradient experiments in which the gradient volume is *not* held constant are at best misleading. The influence of velocity on the separation of the test proteins during a 2-min gradient is illustrated in Fig. 5. Clearly, resolution was directly related to mobile phase velocity (and gradient volume) rather than to column efficiency. Nearly identical results were seen with a C18-250Å-20 μm granular silica gel packing (160 m^2/g surface area), strongly suggesting that this is a general phenomenon (Fig. 6).

3.4. Frontal uptake of lysozyme

The gradient elution data demonstrated that high velocity analytical reversed-phase protein separations are possible. However, a question that must be addressed is whether the proteins had penetrated the particles, or if the adsorption was pellicular. One accepted experimental ap-

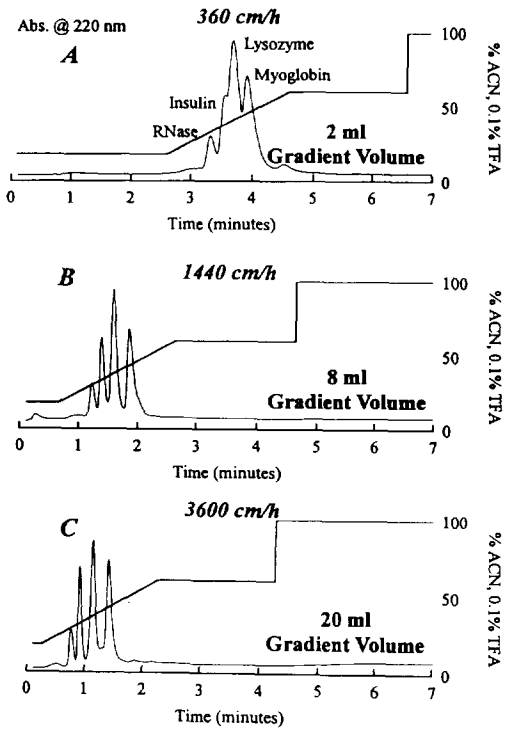

Fig. 6. Influence of velocity on resolution using a C18-250Å-20 μm granular silica gel. Experimental conditions as in Fig. 5.

proach to investigate this issue is frontal adsorption chromatography [15]. During such experiments, solute (lysozyme, 2.5 mg/ml) is pumped through a packed column at various flow rates until it saturates the stationary phase and appears in the column effluent. The column adsorption capacity can then be determined by:

1. Measurement of the mobile phase volume which passed through the column at breakthrough (ca. 10% of feed).
2. Multiplication of this volume by the feed concentration.
3. Division by the column volume.

At slower mobile phase velocities, the solutes have sufficient time to adsorb on the accessible surface area. In experiments at higher velocity, the frontal adsorption capacity would be expected to decrease, if diffusion was limited and the surface area could not be accessed. However, this was not the case for the frontal adsorption of lysozyme on the C18-300Å-15 μm silica (see Fig. 7). The frontal capacities were 25 and 23 mg/ml at 220 and 3600 cm/h, respectively. These data are equivalent given an experimental error of $\pm$ 2 mg/ml. In addition, the breakthrough curves at higher velocities remained relatively sharp (further) demonstrating that lysozyme indeed penetrated the pores.

3.5. Diffusion path length, residence time and mean square displacement

Intuitively, one would not expect the frontal adsorption of lysozyme to be so insensitive to flow velocity. Indeed, much of the recent literature on higher speed protein and peptide sepa-

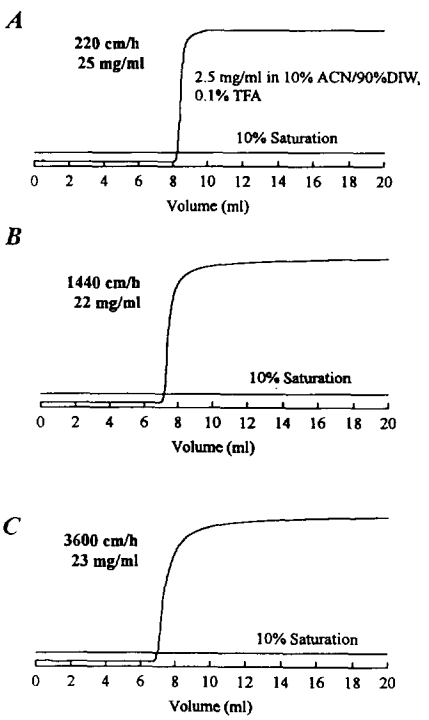

Fig. 7. Lysozyme frontal uptake. Lysozyme frontal uptake curves at 220, 1440 and 3600 cm/h are illustrated in Figs. A–C. Experiments were conducted by pumping a 2.5 mg/ml solution of lysozyme (in 10% ACN with 0.1% TFA) through a 5 × 0.46 cm column packed with C18-300Å-15 μm. Using 10% of feed absorbance (UV 290 nm) in the effluent as the end-point, adsorption capacities of 25, 22 and 23 mg/ml column volume were determined.

rations has suggested that this was not possible [1–5]. However, Kopaciewicz et al. [15] showed the uptake of α-lactalbumin (M_r 17 500) on 10 and 20 μm anion exchange packings with a 500 Å pore diameter to be velocity insensitive, so this result is perhaps not so surprising.

Although not a rigorous description of the processes involved in the solute mass transfer within a column, comparison of the diffusion path length, solute residence time and mean square displacement is instructive. At the very least, this approach provokes thought toward an explanation of the scientific data. The distance from the center of interstitial space to the center of the 15 μm particle (ignoring pore tortuosity) is in the order of 10.5 μm. Furthermore, an unretained solute spends a certain length of time in the column, given by the product of the column porosity (0.6) and the ratio of the column volume (0.83 ml) to the flow rate (0.165 ml/s). In this case, the solute requires around 3 s (residence time) to traverse a 5 × 0.46 cm column packed with C18-300Å-15 μm spherical silica at a linear velocity of 3600 cm/h. In 1905, Einstein derived an equation which related time (t) to both distance and diffusion coefficient:

$$t = \frac{\langle x^2 \rangle}{2D}$$

where x is the diffusion distance and D is the diffusion coefficient [12]. Solving the equation for time, using 10.5 μm as the path length and the diffusion coefficient for lysozyme of 11.5×10^{-7} cm^2/s, results in a value of 0.49 s. Such an analysis obviously assumes that the intra-particle diffusion coefficient of lysozyme (within the 300 Å pore) is similar to that seen in bulk solution. The assumption is reasonable taking into consideration the relative similarity of the lysozyme frontal uptake curves at 360 to 3600 cm/h (see Fig. 7). Given the residence time in the column of 3 s, this implies that a solute molecule could reach the center of the particle in the same time as it would take to traverse about 1/6th of the column length. At a lower velocity of 1440 cm/h, the protein could reach the center of the particle by the time it traveled 1/15th of the bed length, which in this case is equal to only 3 millimeters down a 50-mm bed. This hypothesis is admittedly an oversimplification. Nevertheless, it does provide an interesting perspective as to why high speed separations are possible on conventional particles. Namely, the diffusion of small proteins (i.e. less than ca. M_r 20 000) and peptides is apparently rapid and relatively unhindered across the dimensions of ca. 300 Å, 15 μm chromatography particles.

3.6. Bandwidth

The distances traveled within the column suggest that the eluted protein bandwidths would be very large. Under the normal solvent elution conditions for reversed-phase protein separations, the typical "small molecule" would have a diffusion coefficient is in the range of 5–10 times larger than that of lysozyme. As such, this implies that the protein elution bands would be broader than that of a small organic compound, but only up to an order of magnitude in comparison. However, the isocratic retention of large molecules tends to change dramatically as a function of solvent composition. During gradient chromatography, biomolecules tend to remain tightly adsorbed to the surface until the critical strong solvent condition is achieved, at which point they are desorbed over a very narrow change in solvent strength. In fact, steep gradients may actually have a band sharpening affect especially at high velocity where desorbed solutes are rapidly swept through the (short) column. Consequently, for such selectivity dominated separations (which require few theoretical plates), elution bandwidth is likely to be more related to gradient shape than the distribution of solute within the column, under non-overload conditions. Obviously, the situation is inverse for those separations which require efficiency.

3.7. Influence of velocity on loadability

As stated in the introduction, the goal of preparative chromatography is to isolate a quantity of pure product. As such, separation speed must not come at the expense of loading.

A packing which can be operated at four times the speed, but has one-fifth the capacity is no bargain. To determine the influence of mobile phase velocity (*at constant gradient volume*) on loadability, increasing mass loads of the protein mixture were eluted using a 20 or 2-min gradient at 360 or 3600 cm/h, respectively (Figs. 8 and 9).

There appeared to be only a minimal difference in the resolution of the major components with velocity, especially at higher mass loads. This is because the thermodynamic band broadening due to overload becomes large compared with the kinetic effects, which are best seen at lower loads. Further inspection of these chromatograms reveals that at high speed the resolution of minor components in the protein

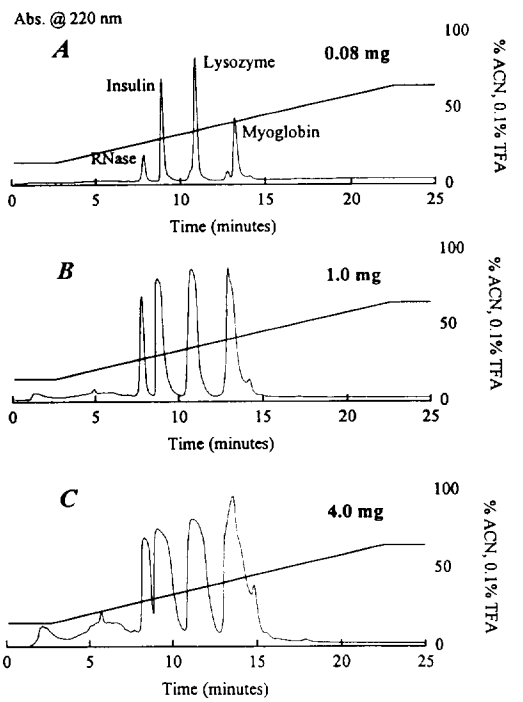

Fig. 8. Resolution vs. load at conventional linear velocity. Chromatograms A–C illustrate the gradient elution profiles of ribonuclease A, insulin, lysozyme and myoglobin at various mass loads. Separations were conducted on a 5 × 0.46 cm column of C18-300Å-15 μm at 360 cm/h (1 ml/min) during a 20-min linear gradient from 15 to 65% ACN containing 0.1% TFA. Total protein loads of 0.08, 1 and 4 mg were chromatographed, as indicated. Solutes were detected by absorbance at 220 nm.

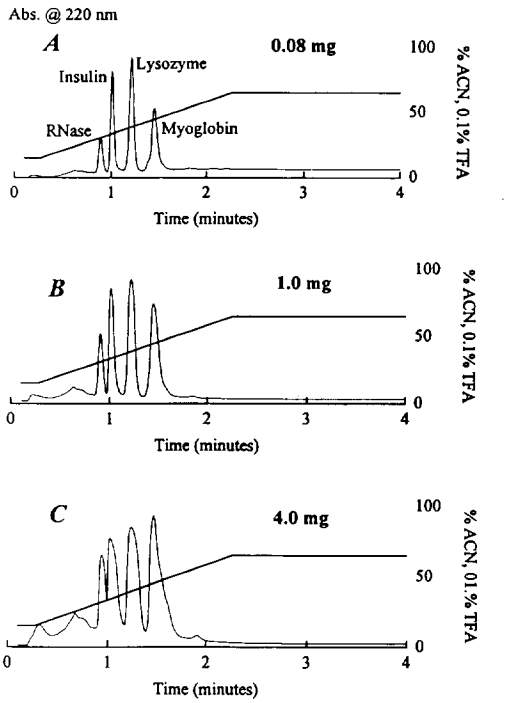

Fig. 9. Resolution vs. load at high linear velocity. Experimental conditions as in Fig. 8, except a velocity of 3600 cm/h (10 ml/min) during 2 minutes.

standards was lost. These data indicate that the ability to increase mobile phase velocity, and thus separation speed is clearly dependent upon separation difficulty. Certainly, for those separations where the adjacent solute bands differ in elution by ca. 3 to 5% acetonitrile, the mobile phase velocity can be increased 5 to 10 fold over "conventional" practice, if the gradient volume is maintained. Increasing the speed for solutes which are less well separated becomes problematic, since such separations are controlled not by the selectivity, but by column efficiency. Under these circumstances, it is necessary to operate at lower flow velocities in order to maximize column efficiency and product yield [10].

3.8. Column pressure analysis

Although smaller particles could be run at high velocity, we chose to investigate materials

of 15 μm. Particles of this size are well established in preparative chromatography as giving an acceptable balance between efficiency and pressure drop. In larger diameter preparative axial compression columns, beds packed with 15 μm spherical silica gels commonly give a reduced plate height of ca. 2.5 which equates to 27 000 plates per meter. In addition, a 15 μm packing will develop a pressure drop just under half that of a 10 μm bead and therefore can be operated at twice the flow rate for a given operating pressure. Since high velocity operation involves increased column pressure drops, it is of value to re-visit the engineering tradeoffs. A more detailed analysis can be found in Ref. [16]. The pressure drop across a packed bed is described by an equation [17] derived from Darcy's law:

$$P = \frac{\phi \eta L u}{d_p^2}$$

where P is the pressure drop, d_p the particle diameter, L the length, u the flow velocity, η the solvent viscosity and ϕ is the resistance parameter (500–600 for spherical particles and 1000 for irregular media). Column pressure is thus linearly related to length and flow rate, and inversely to the square of the particle diameter. Table 2 attempts to provide some perspective regarding the anticipated pressures as a function of length and velocity for spherical monodispersed 15 μm particles. The calculated values should be considered as guidelines.

Short beds have a lower pressure drop at a given flow rate, but also less operational loadability. The longer the bed, the larger the volume and therefore mass load, albeit (for a given velocity and particle diameter) at the expense of pressure and a gain in efficiency. When operated at constant flow velocity, the columns of Table 2 are equivalent in terms of throughput, since both the run time and column load are proportional to length. However, this assumes that they all have the same diameter and the shortest column has a sufficient number of plates to perform the separation. If we consider that the operating pressure is the limiting factor, then it is clear from Table 2 that the 5 cm column can be operated at a flow rate four times that of the 20 cm column. In this case, again provided that the separation can be achieved on the short column, the production rate of the 5 cm column (per unit column volume) is also 4 times that possible using the 20 cm column. Given that real world difficult separations tend to be the norm, this situation is rarely encountered. When working within the practical limits of maximum operating (ca. 1000 psi at large scale), high flow rates can only be achieved with short bed (or larger particles) which concomitantly results in a plate number reduction. Once the efficiency required for the separation is established and the Knox parameters are known, it is relatively easy to calculate for any given maximum operating pressure what is the highest possible flow rate together with the suitable column and particle dimensions. Obviously, this also assumes that the selectivity of the packing material does not appreciable change as a function of particle size. In practice, therefore, the extra production rate possible with short

Table 2
Calculated column pressures for 15 μm spherical particles

Column length (cm)	Column pressure (p.s.i.) at indicated velocity[a]			
	1000 cm/h	2000 cm/h	3000 cm/h	4000 cm/h
5	56	112	168	224
10	112	224	336	448
15	168	336	504	672
20	224	448	672	896

[a] Assuming $\phi = 500$ and $\eta = 1$ (mPa · s).

columns operated at high velocity is often offset by the loss of column efficiency relative to longer columns. For the present purpose, it can be argued that a relatively easy separation can be achieved with high productivity using a column of perhaps 1500 plates which would be 15 to 20 cm in length and packed with 15 μm particles. This column could be run at flow velocities of 1000 to 2000 cm/h (corresponding 330 to 660 ml/min on a 50 mm I.D. column) and would require up to 600 p.s.i. (1 p.s.i. = 6894.76 Pa) depending on the system (extra-column) pressure.

3.9. Other considerations

It is not the intent of this paper to suggest that every reversed-phase protein and peptide separation currently conducted on conventional media should be accelerated. However, the results do indicate that speed should be one of the variables considered in the optimization of preparative separation processes. In reality, there are other operational issues which also require consideration when optimizing separation speed. For example, during a 2-min gradient separation carried out at 3600 cm/h, the band widths may be as small as 18 s. At best, this may complicate accurate fraction collection. An additional factor is that of temperature effects within the columns [18]. As flow rates are increased, the operating pressure also increases and the effects of solvent frictional heating become appreciable. At very high velocities, radial temperature gradients can be established which may distort peak shape. This would have a negative impact upon the purity–recovery relationship. In practice, the separations are probably better operated at more modest velocities. Subsequently, our example above may be more realistically run at 1440 cm/h with a 5-min gradient. Under these conditions, the typical peak width would be approximately 45 s, frictional heat is of little concern and the productivity is still enhanced by a factor of four over "conventional" operation at ca. 360 cm/h.

4. Conclusions

The results clearly demonstrate that high velocity protein and peptide chromatography can be conducted on a conventional C18-300Å-15 μm packing. The separation of ribonuclease A, insulin, lysozyme and myoglobin was achieved in 90 s at a mobile velocity of 3600 cm/h during a two-min linear gradient. However, the ability to conduct such a high speed separation resulted more from the high inherent chromatographic selectivity observed for this separation, rather than any unique physical property of the matrix. Since the elution of adjacent protein bands differed by about 3 to 5% acetonitrile, the separation was selectivity controlled and few theoretical plates are required. This allows mobile phase velocity to have a positive influence on resolution as more eluent can be interposed between eluting peaks.

The frontal uptake capacity of lysozyme on a 5×0.46 cm column was constant within experimental error at linear velocities ranging from 220 to 3600 cm/h. These data demonstrate that the adsorption of small proteins into packings with pores in the order of 300 Å was velocity insensitive. Comparison of the diffusion coefficients with particle geometry suggested that the diffusion of a small protein such as lysozyme is reasonably rapid and unhindered across the dimensions of a ca. 300 Å, 15 μm HPLC particle.

Loading studies using the four proteins gave similar results at both 360 and 3600 cm/h. For the separation of major components, this represents a 10-fold increase in column throughput. However, the resolution of minor components, which represented a more difficult separation (requiring more theoretical plates), was compromised.

In situations where selectivity dominates and the separation can be justifiably accelerated, 15 μm particles offer a favorable balance between efficiency and pressure of operation. A 20 cm bed of 15 μm spherical particles has a (calculated) pressure drop of ca. 450 p.s.i., when run at 2000 cm/h with a mixture of acetonitrile and

water. This value is well within the limits of preparative HPLC systems.

With the current emphasis in high speed separations being placed upon new particle configurations, it is important to be familiar with the performance available from what might be considered conventional particles (mono-modal pore distribution). Certainly, it is clear that under certain conditions the "conventional" reversed-phase chromatography of small proteins and peptides can be substantially accelerated, thus increasing throughput without incurring losses in resolution and loadability. Therefore, speed should be exploited when appropriate. Nevertheless, our conclusions are somewhat circular in that they return to one of the most quintessential paradigms of chromatography, i.e., whereas easy separations can be run fast, difficult ones must walk . . .

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