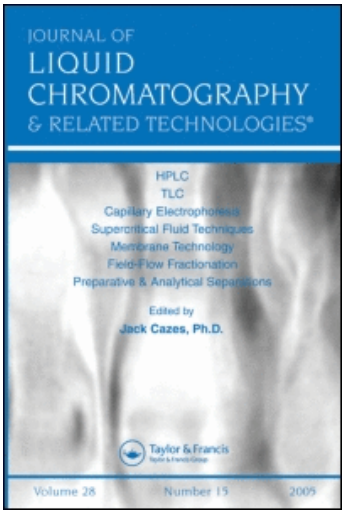

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Protein Loading Capacity and Textural Properties of Column Packings in Reversed-Phase HPLC

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To cite this Article Kim, Young S. , Sands, Bruce W. and Bass, Jonathon L.(1987) 'Protein Loading Capacity and Textural Properties of Column Packings in Reversed-Phase HPLC', Journal of Liquid Chromatography & Related Technologies, 10: 5, 839 — 851
To link to this Article: DOI: 10.1080/01483918708066739
URL: http://dx.doi.org/10.1080/01483918708066739

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(5), 839-851 (1987)

PROTEIN LOADING CAPACITY AND TEXTURAL PROPERTIES OF COLUMN PACKINGS IN REVERSED-PHASE HPLC

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ABSTRACT

The relationship between the textural properties (pore size, pore volume and surface area) of reversed-phase silica gel packings for HPLC and the dynamic loading capacity of large biomolecules was studied by using silica gels manufactured by similar processes. Several silica gels whose unbonded pore diameters range from 100 to 250 A and whose pore volumes range from 1.0 to 1.4 ml/g have been prepared and characterized. The bonded phase is monomeric C18. The textural properties of the bonded silica gels are also presented and related to the properties of the unbonded silica gels. Chromatographic evaluation with typical proteins in an underload-to-overload condition was performed in order to relate the influence of textural properties of silica gel to loading capacity and resolution. The packings with larger pore size and pore volume produced better column performance and higher loading of proteins.

INTRODUCTION

The relationship between the resolution of small organic molecules and proteins vs. pore size of C18 reversed-phase silica gels has been described earlier (1). It was found that the resolution of proteins increases while that of small molecules decreases as the pore diameter and pore volume of the silica gel increases.

In protein separations, the use of large pore (>300 A) silica gel packing materials has been generally suggested (2, 3, 4). However, the effects of pore size for reversed-phase HPLC columns have not been well clarified in the literature, especially under sample overloading conditions.

A number of recent studies regarding resolution of protein samples and efficiency as well as sample loading and recovery have been reported (5). However, since the properties of the materials such as surface area, pore diameter, pore volume, etc. were not systematically controlled, the interpretation of results was complicated and unclear. Another significant problem has been the lack of materials whose basic chemistry and surface modification are similarly controlled.

In this study, we prepared several well characterized silica gels from similar base materials and with similar surface modification procedures for C18 reversed-phase bonding. The surface properties are well characterized and the relationship between the pore diameter and pore volume is essentially linear.

These well-defined materials will enable us to understand the relationship of the above parameters to sample loading capacity and peak resolution in large biomolecule separations.

MATERIALS AND METHODS

Materials

The treatment of silica gels and the procedures for C18 reversed-phase bonding and column packing have been described previously (1). Particle size analysis was by HIAC. Their physical properties are summarized in Table 1.

The solvent system for protein separation is a gradient mode consisting of (A) 0.1% trifluoroacetic acid (TFA) in 95% acetonitrile and (B) 0.1% TFA in H_2O. The linear gradient slope runs from 0% (A) to 75% (A) in (B) over a period of 30 minutes and the flow rate is 1 ml/min. The sample mixture used to evaluate resolution and efficiency contains ribonuclease A (M.W. = 13.7 K), insulin (M.W. = 5.4 K), lysozyme (M.W. = 14.3 K), bovine serum albumin (BSA) (M.W. = 65 K) and ovalbumin (OVA) (M.W. = 43 K), (all from Sigma Chem.) was prepared by dissolving these in solvent B.

Uracil was used as a marker for t_o measurement. For overloading tests, 250 mg each of bovine serum albumin and ribonuclease A was dissolved in 5 ml of solvent B and the sample loading amount was determined by adjusting the sample injection volume.

TABLE 1

PHYSICAL PROPERTIES OF COLUMN PACKINGS

SILICA GEL	100 A	200 A	250 A
Surface Area (m^2/g)	423	261	204
Pore Volume (ml/g)	1.13	1.30	1.36
Pore Diameter (A)	105	188	250
C18-BONDED SILICA GEL	**100 A**	**200 A**	**250 A**
Surface Area (m^2/g)	176	147	136
Pore Volume (ml/g)	0.53	0.80	0.93
Pore Diameter (A)	85	162	216
Carbon Content (%)	19.20	15.47	13.20
Silane Concentration ($\mu mol/m^2$)	2.52	3.09	3.25
d_v50 (μm)	21.6	21.1	22.5
d_v90/d_v10	2.2	2.1	2.2

Apparatus

The chromatographic system was a Waters dual HPLC pump model 510 with a gradient controller, a Model 481 UV absorption detector, and a Valco loop injector. Data acquisition and calculations were obtained by an IBM 9000 microcomputer with a chromatographic applications program.

RESULTS AND DISCUSSION

The column performance of the three packings (Table 1) using the five-protein mixture before performing the overloading test is shown in Figure 1. The peak shape and resolution with large pore packings (216 and 162 A)

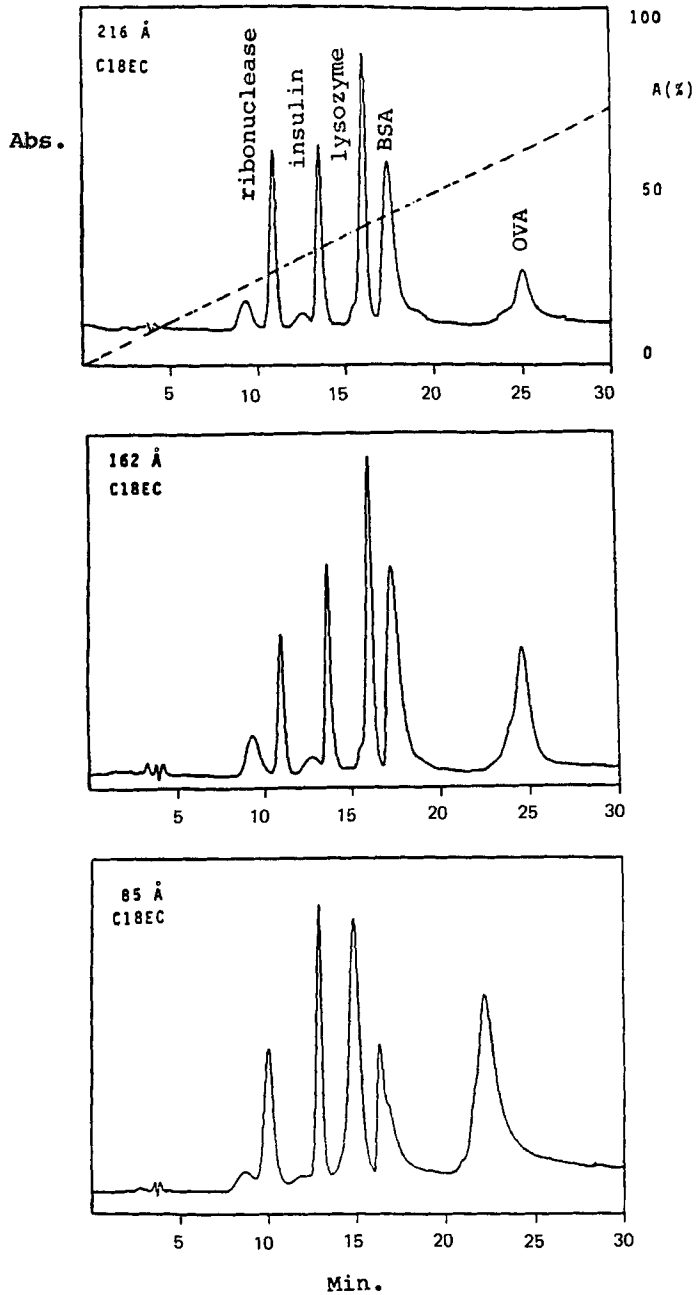

Figure 1. Column performance before overloading tests. Dotted line is profile of gradient elution.

are significantly better than with the small pore packing (85 A). However, the difference in performance between the packings whose pore diameters are 216 A and 162 A seems to be insignificant. The retention time and resolution of peaks become smaller with small pore packings. Column overloading with the two protein mixture as shown in Figure 2 shows also that the large pore packings give narrower and better resolution of peaks.

The impurity peak in BSA is clearly visible under the overloading condition with the 216 A packing. In contrast, the BSA peak shape is rapidly degraded by increasing the sample loading on the 85 A packing The resolution of ribonuclease A and BSA is good up to a loading of 20 mg each per gram of packing on the wide-pore columns, but this is not the case for the 85 A packing. Even at low sample loading, the BSA peak is broad and tailing with the 85 A packing. After these overloading tests, the column efficiency was tested again with five standard proteins as shown in Figure 3. The column performance of the 216 and 162 A packings did not change noticeably. However, the column with 85 A packing shows a significant peak distortion and tailing between the BSA and OVA peaks. These large molecules may be adsorbed in small pores tightly and desorbed slowly due to steric effects.

There appears to be a direct relationship between the resolution and the pore size of the packing material as shown in Figure 4. For a given protein loading level resolution increases as the pore size increases, but this effect is less pronounced at higher protein loadings. This relationship is presented in another fashion in Figure 5. When the sample loading increases,

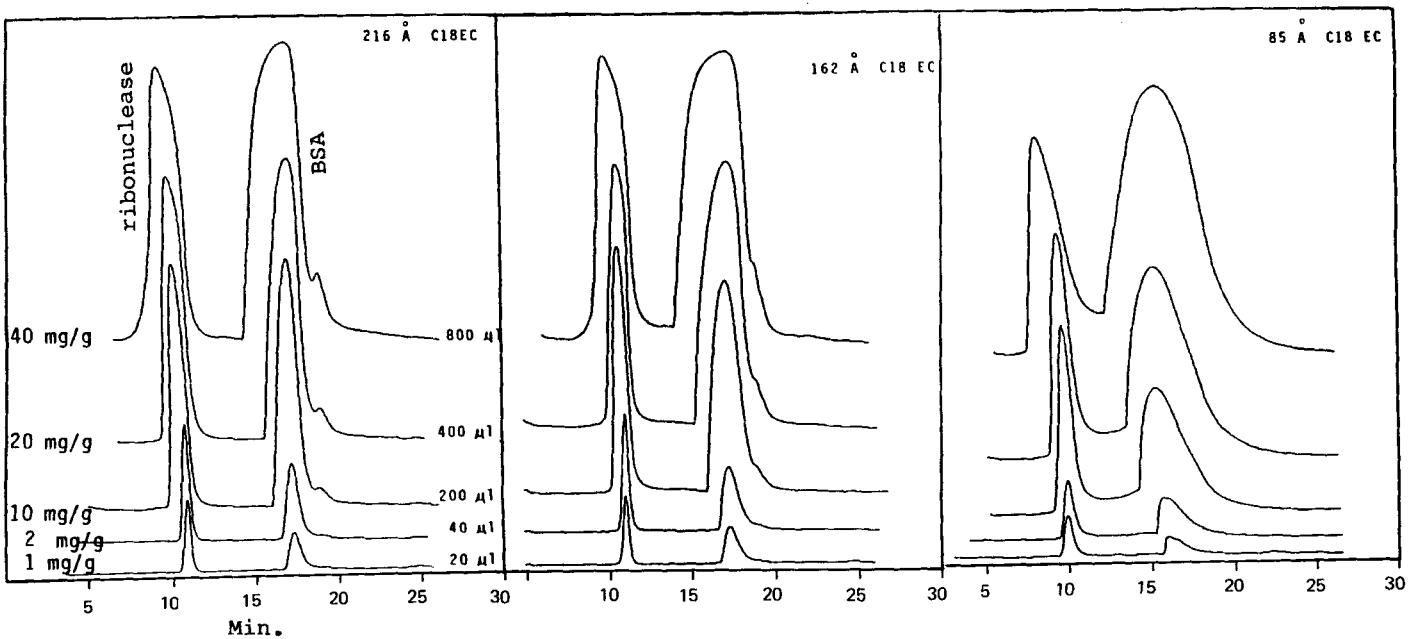

Figure 2. Chromatograms of sample overloadings.

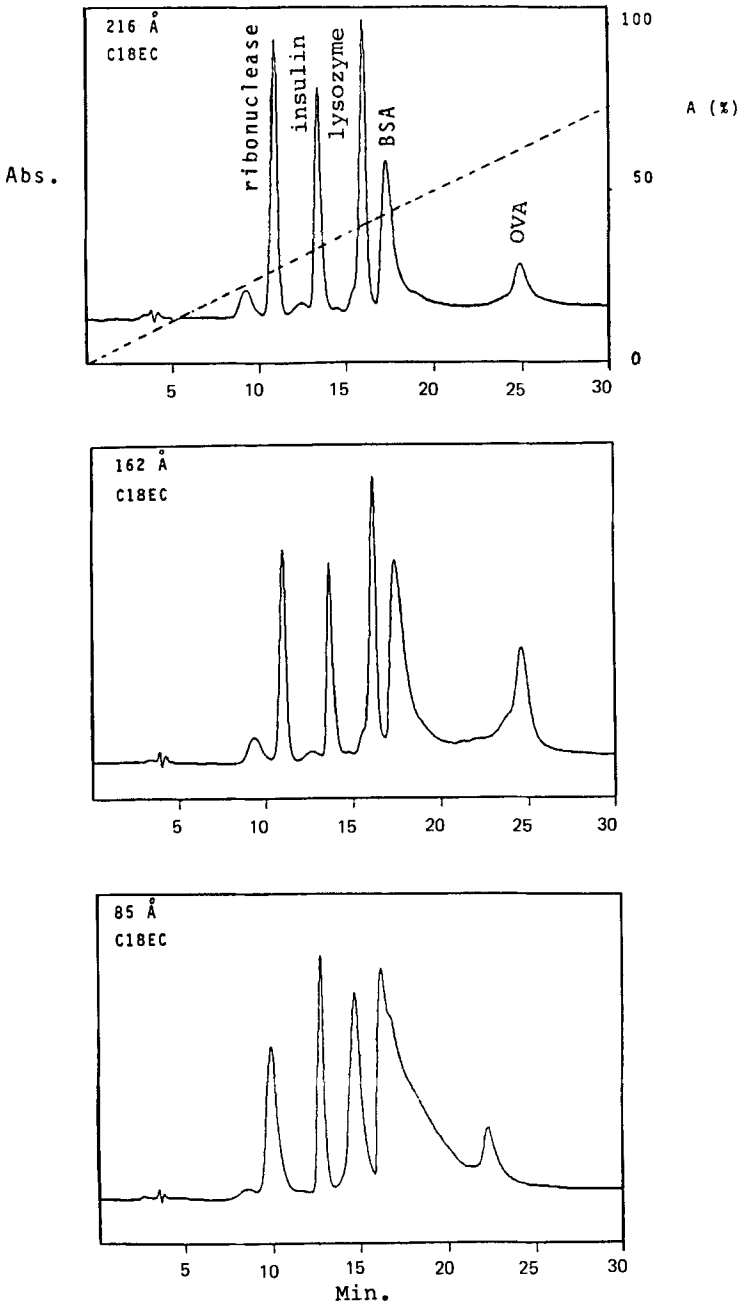

Figure 3. Column performance after overloading tests.

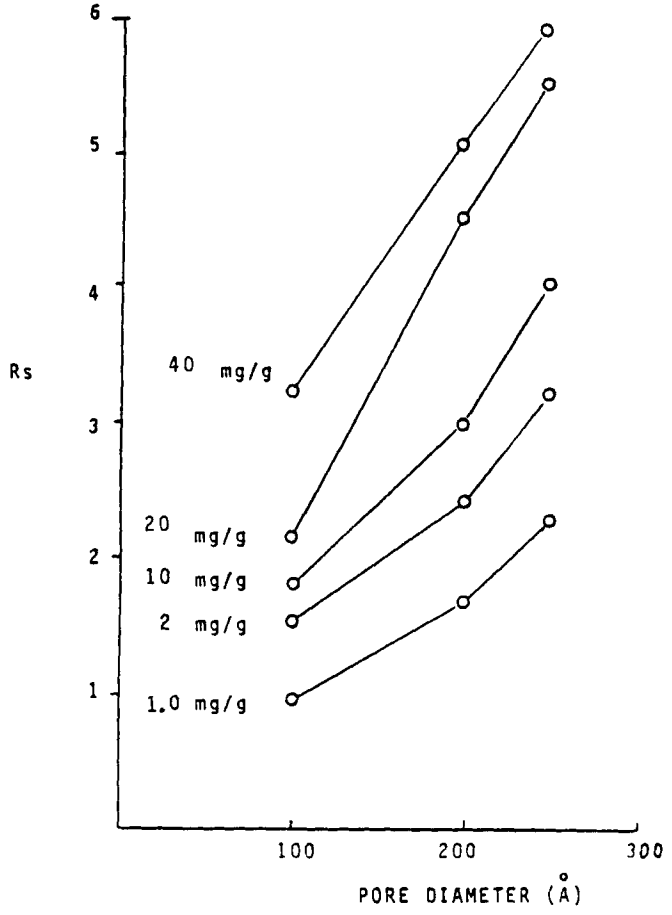

Figure 4. Resolution vs. pore diameter of reversed-phased supports at different sample loadings.

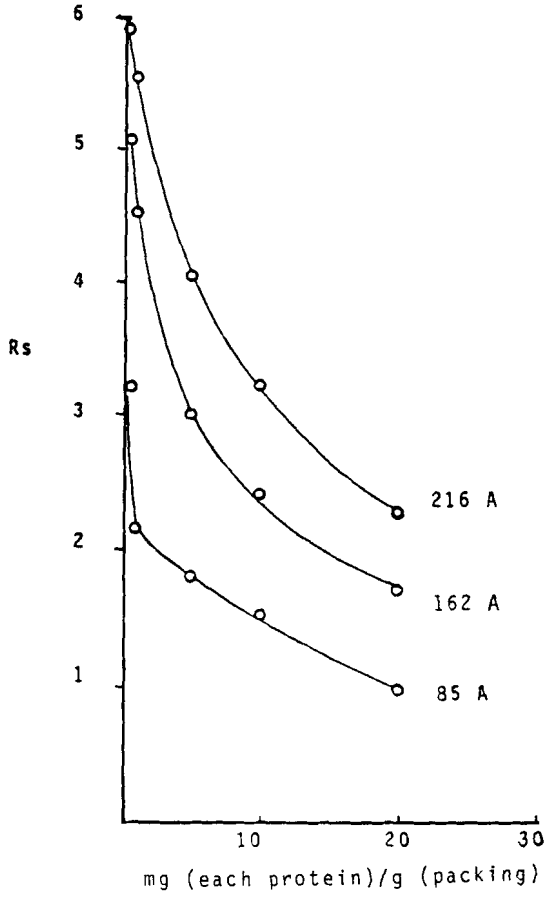

Figure 5. Resolution vs. protein loadings with different pore diameter reversed-phase packings.

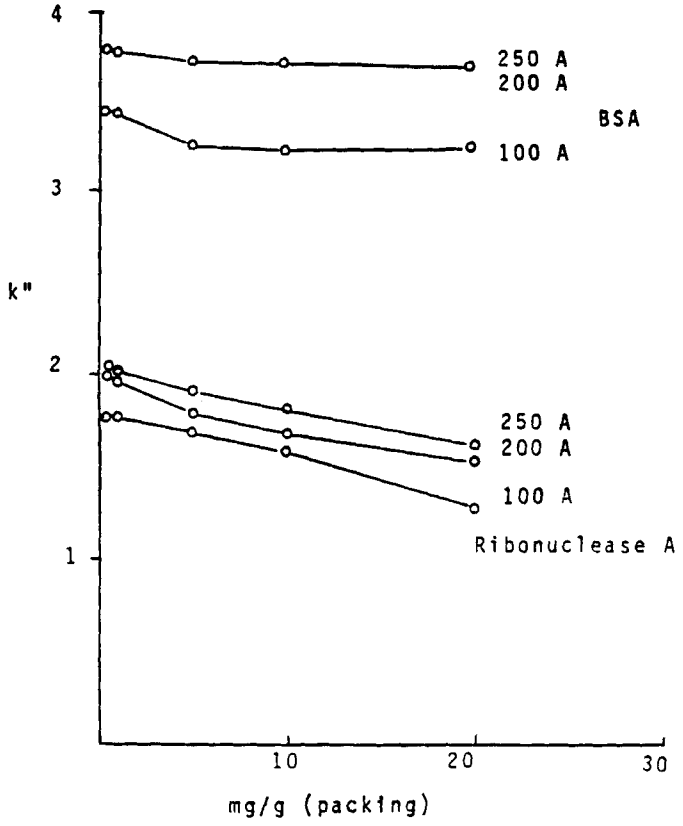

Figure 6. Retention parameter vs. protein loadings with different pore diameter reversed-phase packings.

the resolution decreases more rapidly with small pore packings.

The k" of Figure 6 is defined as

$$k"_i = [t_i(g) - t_o(g)]/t_o(g)$$

and is comparable to the commonly used retention parameter, k', in the isocratic elution mode. Since k' is not strictly applicable to a gradient system and in

particular a sample overloading situation, k" is used as the retention parameter to quantify the change of retention under identical gradient elution conditions for different pore size packings. The columns with large pore size packings show a smaller change in k" as the protein loading increases.

Although the apexes of two peaks are pointing in opposite directions, the position of each peak is shifting toward earlier elution as the sample loading increases. This is a volume overloading situation; however, the peak shape and shift show more similarity with a concentration overloading effect (6).

CONCLUSIONS

The resolution of two proteins, ribonuclease A and bovine serum albumin increases proportionately to the pore diameter (up to 250 A) and pore volume of C18 reversed-phase column packings. The protein loading capacity is lower on high surface area supports in contrast to previous observations for small molecules. This occurs because most of the surface area in such materials is in small pores which may be blocked by adding the bonded phase or are too small for free access of the proteins for adsorption/desorption. Therefore, column performance decreases more rapidly with small pore packing materials. Also, wide pore diameter and high pore volume supports show a smaller decrease in the retention parameter as sample loading increases.

ACKNOWLEDGEMENTS

We wish to thank Virgil Swadley, Beverly Tait and Charlotte Reynolds for their dedicated contribution to this work.

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