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HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY. PORE-SIZE EFFECTS

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SUMMARY

The efficiencies and capacities of diol-bonded silica packings of pore size 60–4000 Å were examined. Glucosamine was bonded to the diol phase and affinity chromatographic separations of concanavalin A and bovine serum albumin were performed Plate heights were smallest when silica of very small pore size (such that proteins were totally excluded) or very large pore size was used. Separations of the two proteins could be achieved in 1 min or less

Two factors were responsible for the poorer efficiency at intermediate pore sizes Restricted diffusion in the pores caused a large increase in the plate height when the pore diameter was similar to the protein molecular diameter, in agreement with theory. Slow adsorption–desorption kinetics contributed to band broadening when the eluent was not strong enough to reduce k' to zero

Protein adsorption capacities were strongly affected by pore size since surface areas of silica are inversely related to the diameter of the pores

INTRODUCTION

High-performance affinity chromatography (HPAC) offers the possibility of separating complex samples with a selectivity which cannot be achieved by any other chromatographic method This selectivity is derived from the use of bonded stationary phases consisting of some specific biochemical, the affinity ligand. Ideally, a sample passed through an HPAC column should separate into two bands. The first band should elute with capacity ratio $k' = 0$ and contain all the compounds which do not bind to the affinity ligand The second band, containing only the analyte, should be strongly adsorbed to the ligand and should not elute in reasonable time periods under isocratic conditions Increasing the mobile phase strength by a change in pH or other parameter then causes the analyte peak to elute.

Analytical applications of HPAC require that only the analyte be retained on the column (*i e.* high selectivity, low non-specific adsorption) and that the separation take place in a minimum amount of time (*i e.* short retention times, high chromatographic efficiencies). The latter requirement will be examined in this paper as it is affected by the pore size of the support material to which the affinity ligand is attached.

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Pore diameters of 60–4000 Å have been used in previous HPAC applications[1–5]. The surface areas of silica-based supports are inversely proportional to the pore diameter. However, when the pores are too small for the analyte to penetrate, the effective surface area vastly decreases. One effect of changing the pore diameter will, therefore, be to alter the adsorption capacity of the column.

Typical analytes in HPAC are large biochemicals with small diffusion coefficients[4]. The efficiency of HPAC columns should improve if the analyte cannot diffuse into stagnant mobile phase in the pores. Further, diffusion is restricted when the size of the pore and the size of the analyte are similar[6]. The kinetics of the adsorption and desorption processes may also affect the efficiency[4].

A model system consisting of D-glucosamine, immobilized via the amino group, and the lectin concanavalin A (Con A) was chosen for this investigation. Specific elution of Con A was accomplished by adding methyl α-D-mannopyranoside to the mobile phase[7]. Under the conditions used here, Con A was present largely in the form of a dimer[8] of molecular weight 54,000[9]. Bovine serum albumin (BSA), molecular weight 68,000[10], was chosen to measure efficiencies in the absence of any retention.

EXPERIMENTAL

Reagents

All biochemicals were obtained from Sigma (St. Louis, MO, U S A.) and were the purest grade available. 10 μm LiChrosorb Si 60 and LiChrospher Si 100, 500 and 4000 were obtained from Rainin (Woburn, MA, U S.A.). (3-Glycidoxypropyl)trimethoxysilane was obtained from Petrarch (Levittown, PA, U.S.A.). 1,1′-Carbonyldiimidazole came from Aldrich (Milwaukee, WI, U.S A.).

Apparatus

A Model 334 gradient liquid chromatograph (Beckman, Berkeley, CA, U.S A.) was modified by replacing the mixing chamber with a T-connector and by placing a guard column between the T and the injection valve. A Beckman Model 155 variable wavelength absorbance detector was modified by reversing the connections so that the heat-exchanger was after the flow-cell. A Hewlett-Packard (Avondale, PA, U.S.A.) 3390A integrator was used for peak area measurements. A Haskel air-driven pump (Alltech, Deerfield, IL, U.S.A.) and a Model 705 stirred-slurry column packer (Micromeritics, Norcross, GA, U S.A.) were used for column packing.

Methods

Diol-bonded phases were synthesized by a modification of previous methods[1 11] using the quantities shown in Table I. The silica was shaken for 5 h at 90°C with the buffer and silane. After filtering and washing with water, the epoxide groups were hydrolyzed by refluxing 1 h in pH 3 sulfuric acid.

After washing and drying, the diol-bonded silica was activated[12] by shaking for 30 min at room temperature with the amounts of 1,1′-carbonyldiimidazole and dioxane given in Table I. After washing with dioxane, the activated silica was shaken for 22 h at room temperature in a pH 8.0 solution of glucosamine · HCl (amounts given in Table I) in a stoppered test tube which had been flushed with nitrogen. The

TABLE I

SYNTHESIS OF STATIONARY PHASES

		Pore size of silica (Å)*			
		60	*100*	*500*	*4000*
Diol synthesis	Wt silica (g)	1 0	1 0	1 0	1 0
	Vol 0 1 *M* sodium acetate, pH 5 5 (ml)	15	10	4 0	5 0
	Vol (3-glycidoxypropyl)trimethoxysilane (ml)	0 75	0 50	0 10	0 015
Activation and coupling	Wt. diol-bonded silica (g)	0 65	0 60	0 60	0 60
	Diol content** (μmole)	650	300	120	9
	1,1 -Carbonyldiimidazole (μmole)	6500	3000	1200	350
	Vol dioxane (ml)	13	6 0	2 4	2 0
	Glucosamine HCl (μmole)	3250	1500	600	500
	Vol glucosamine solution, pH 8 0 (ml)	13	6 0	2 4	2 0

* Given by the manufacturer E Merck Darmstadt, G F R

** Determined by periodate titration[13]

derivatized silica was washed with 2 *M* sodium chloride solution and water, then slurry-packed into 5 cm × 4 6 mm columns at 3000 p s i using the weak buffer below

Chromatography was performed at room temperature The Con A solutions were kept on ice to prevent precipitation. The weak mobile phase was 0.2 *M* sodium chloride, 0 05 *M* sodium phosphate, pH 6.0 Uracil (0 016 mg/ml), BSA (10 mg/ml) and Con A (10 mg/ml) were prepared in the weak buffer. The Con A solution also contained 1-m*M* concentrations of $MnCl_2$ and $CaCl_2$. The strong mobile phase contained 0.02 *M* methyl α-D-mannopyranoside in the weak buffer. Detection was performed at 280 nm

RESULTS AND DISCUSSION

Column efficiency for BSA

Chromatographic efficiency was assessed using the height equivalent to a theoretical plate, *H*. *H* was calculated using the peak width at half-height[14]. The calculations did not take into account peak tailing[14] which would have increased the size of *H*. Variations in the silica particle size distribution, particle shape, and packing procedure contributed variability to the values of *H* determined for various columns. The values presented should, therefore, be taken as approximate values only

Figs 1 and 2 show the efficiencies for uracil, BSA, and Con A at 0 2 and 1 0 ml/min, respectively. The plate height values for the small, unretained solute uracil ranged from 35 to 46 μm at 0.2 ml/min These were "good" columns since the plate heights were in the range of 2 to 10 particle diameters[15]. The plate heights increased moderately at 1.0 ml/min

The efficiencies for the unretained protein BSA varied dramatically as the pore size changed. The reason for this can be explained using Table II, which lists the total porosity, ε_{tot}, of each column The total porosity is the fraction of the column volume which the solute can penetrate[14] Comparisons of the total porosities for uracil, BSA, and a large, totally excluded protein indicate that BSA was totally excluded from the

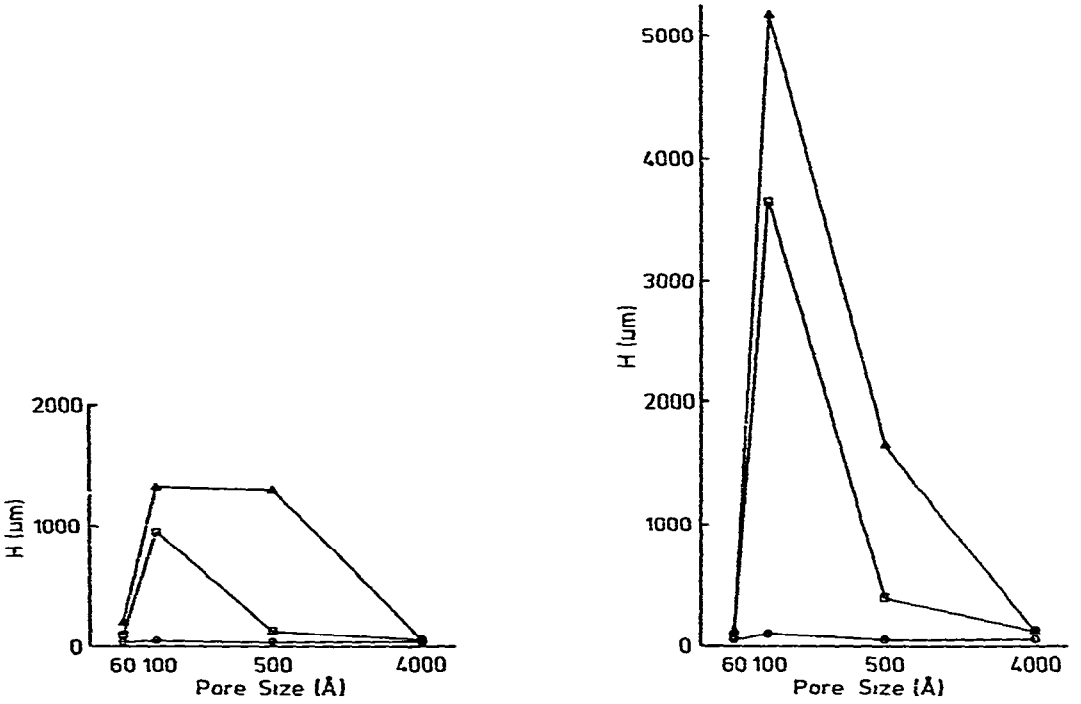

Fig. 1 Plate height *vs* log pore size at a flow-rate of 0 2 ml/min. Uracil (○) and BSA (□) solutions were injected into the weak mobile phase. Con A (△) was injected into the strong mobile phase

Fig. 2. Plate height *vs* log pore size for uracil (○), BSA (□) and Con A (△) at 1 0 ml/min.

pores of the 60-Å support, partially excluded from the 100-Å pores, and mostly included in the 500- and 4000-Å pores.

Consider the following plate height equation for a non-retained solute on a "good" column[14]:

$$H = \frac{u^{0\,33} d_p^{1\,33}}{D_m^{0\,33}} + \frac{2D_m}{u} + \frac{0\,05 u d_p^2}{D_{sm}} \qquad (2)$$

TABLE II

TOTAL POROSITY, ε_{tot}

	Pore size (Å)			
	60	*100*	*500*	*4000*
Uracil	0.84	0 90	0 88	0 82
BSA	0.50	0 62	0 75	0 80
Large protein	0 51*	0.40**	–	–

* Immunoglobulin G (human)
** Thyroglobulin (porcine)

D_m and D_{sm} are the inter-particle and intra-particle diffusion coefficients (cm^2/sec), respectively, of the solute in the mobile phase; u is the mobile phase velocity (cm/sec); and d_p is the particle diameter (cm). The second term, the plate height due to longitudinal diffusion, is negligible under the conditions used here.

For the 60-Å pore size column the third term, the plate height due to diffusion in stagnant mobile phase of the pores, was zero for BSA since BSA was totally excluded from the pores. The remaining term, the plate height due to eddy diffusion and mobile phase mass transfer, can be calculated for BSA under the conditions used here $F = 0.2$–2.0 ml/min ($u = 0\ 04$–$0\ 40$ cm/sec), $d_p = 10^{-3}$ cm, and $D_m = 6\ 6\ 10^{-7}$ cm^2/sec (measured in water at 25°C)[16]. The calculations show that H should have increased from 39 to 83 μm in this flow-rate range The observed values were in fair agreement and ranged from 60 to 110 μm, with a minimum at 0 5 ml/min. These values were only 1–3 times as great as the values measured for uracil, hence HPAC columns from which biopolymers are totally excluded exhibit excellent efficiencies

For the remaining three columns both the first and third terms of eqn 1 must be considered The first term was calculated to be nearly constant at about 35 μm for all three columns for BSA. The third term should have been nearly constant at about 25 μm for all three columns if $D_m = D_{sm} = 6.6 \cdot 10^{-7}$ cm^2/sec This clearly does not account for the tremendous increase in H of the 100-Å pore size column (Fig 1) When the molecular diameter of the solute is similar to the pore diameter, diffusion is restricted and D_{sm} decreases[6]. One expression[6] for D_{sm} is

$$D_{sm} = \frac{D_m}{2.1}(1 - 2\ 104\lambda + 2.09\lambda^3 - 0\ 95\lambda^5) \quad (2)$$

where λ is the ratio of the solute diameter/pore diameter

For BSA on the 100-Å pore diameter support, λ was close to 1 0 since BSA was partially excluded. The 74-Å Stokes diameter of BSA in water at 25°C[16] and the somewhat smaller pore size of the support after the bonded-phase was attached support this conclusion. Assuming that λ was equal to 1.0 for the 100-Å support and that λ was inversely proportional to the pore diameter, values of D_{sm} and H were calculated (Table III) for the three columns at 0.2 ml/min using eqns. 1 and 2 The calculated plate height values were in good agreement with the measured values Note that the effect of restricted diffusion decreased rapidly as the pore size increased. The efficiency of the 4000-Å column was not much different from that of the 60-Å column discussed previously.

These results explain why the plate heights for protein separations on size-exclusion columns are often 1 mm or more[17] Restricted diffusion may inadvertently

TABLE III

EFFECTS OF RESTRICTED DIFFUSION AT 0 2 ml/min

Pore size (Å)	λ	D_{sm} *(cm^2/sec)*	*H, calculated (μm)*	*H measured (μm)*
100	1 0	$1\ 1\ 10^{-8}$	1500	950
500	0 2	$1\ 9\ 10^{-7}$	105	120
4000	0 025	$3\ 0\ 10^{-7}$	75	59

affect other types of separations as well. It may have caused the poorer resolution of ovalbumin samples on a 100-Å pore size anion-exchange support compared to a 300-Å support[18] or the larger plate height for BSA on a 100-Å reversed-phase support compared to a 500-Å support[19].

Restricted diffusion is unavoidable in size-exclusion chromatography, but clearly should be avoided in affinity, ion-exchange and reversed-phase separations

Column efficiency for Con A

The plate heights for Con A were measured by injecting Con A solutions into the strong mobile phase, 0 02 *M* methyl α-D-mannopyranoside (Figs. 1 and 2). Since the dissociation constant of the resulting complex was much smaller than that of a Con A–N-acetyl-D-glucosamine complex ($4.7 \cdot 10^{-5}$ *M vs.* 7.2 10^{-3} *M*, respectively[20]), it was anticipated that no retention of Con A would be observed. In that case, since Con A has a molecular weight and diffusion coefficient ($6.2 \cdot 10^{-7}$ cm^2/sec at 20°C in water[21]) similar to BSA, the plate heights measured for Con A should have been very close to those measured for BSA. Figs. 1 and 2 indicate, however, that the efficiencies for Con A were generally much poorer than for BSA, especially on the 500-Å and 100-Å columns. It appeared that the Con A was slightly retained on these two columns, and that the larger plate heights might have resulted from the adsorption-desorption kinetics.

To test this, a 500-Å pore size column was prepared containing diol-bonded silica. Table IV compares the plate heights and retention volumes measured on this column and the 500-Å affinity column. On the diol-bonded column, *H* for Con A was close to that of BSA, as expected. On the affinity column, *H* for Con A increased more than 10-fold over that of the diol-bonded column even though *k'* was only about 0 4.

TABLE IV

COMPARISON OF 500-Å PORE SIZE COLUMNS WITH AND WITHOUT GLUCOSAMINE ATTACHED AT 0.2 ml/min

	Diol-bonded silica		*Affinity column*	
	Retention volume (ml)	*H (μm)*	*Retention volume (ml)*	*H (μm)*
Uracil	0.76	41	0 73	39
BSA	0 64	130	0 62	120
Con A	0.70	120	1 0*	1300

* Measured in the strong mobile phase, 0 02 *M* methyl α-D-mannopyranoside

H was next measured on the 500-Å affinity column while varying the strength of the mobile phase. Table V shows that *H* increased rapidly as the mobile phase strength decreased. Kasche *et al.*[4] also observed *k'*- and flow-rate-dependent changes in *H* when analyzing α-chymotrypsin on immobilized soybean trypsin inhibitor using non-specific elution.

A term describing the plate height contribution of adsorption–desorption kinetics needs to be added to eqn. 1. Equations written for this purpose[4,22] are useful in

TABLE V

EFFECT OF MOBILE PHASE STRENGTH ON *H* FOR THE 500-Å PORE SIZE COLUMN AT 1 0 ml/min

Methyl-α-D-mannopyranoside (M)	*k'* *	*H (μm)*
0 05	0 2	790
0 02	0 4	1200
0 01	1 0	2000
0 005	1 4	2600
0 002	4 3	5100
0 001	11	6500

* Calculated based on a void volume of 0 7 ml measured on the 500-Å pore size diol-bonded column

some cases[4], but do not take into account competitive binding and elution such as was used here The equations do predict that this term will go to zero when $k' = 0$

The practical implications of Tables IV and V are very important Isocratic HPAC separations should be avoided because the analyte may elute as a broad, dilute peak even if it is only weakly retained Gradient elution should always be performed The fastest and most sensitive separations will take place when the strength of the mobile phase is sharply increased such that k' for the analyte drops as close to zero as possible It should be pointed out that a mobile phase which prevents binding to the ligand may not be as effective once the analyte is bound to the ligand[23]

Separation time

A mixture of Con A and BSA was separated on columns of different pore size Because of peak tailing, calculations involving resolution were not adequate to describe the separation. Instead, the separation time was defined here as the retention time of the Con A peak at a flow-rate of 1 0 ml/min when the height of the valley between the BSA and Con A peaks was 10% of the average height of the two peaks

Fig 3 shows the separation on each of the columns All were quite fast The 60-Å column required only 0 7 min, largely because of the small volume accessible to the proteins The 4000-Å column required 1 0 min, while the 500-Å and 100-Å columns took 1 3 and 1 4 min, respectively All the runs are shown with the same time axis but with different full-scale sensitivities The 60-Å and 4000-Å columns were also the most sensitive since the injection of 50 μg of Con A resulted in peak heights of 0 41 and 0.56 absorbance units, respectively, compared to 0 16 and 0 14 absorbance units for the 500-Å and 100-Å columns, respectively

The poorer sensitivity and separation time of the 100-Å column was caused primarily by restricted diffusion in the pores Note how wide the BSA peak was for the 100-Å column relative to the other columns.

Only the Con A peak was broad on the 500-Å column This was due to the adsorption–desorption kinetic problem discussed previously The strong mobile phase was not strong enough to eliminate all retention of Con A. This problem was not observed with the 60-Å and 4000-Å columns because the latter columns contained less available affinity ligand (see below) and thus the 0 02 *M* methyl α-D-mannopyranoside was more effective in decreasing the Con A retention

The separation time, using the above criterion, was also measured as a function

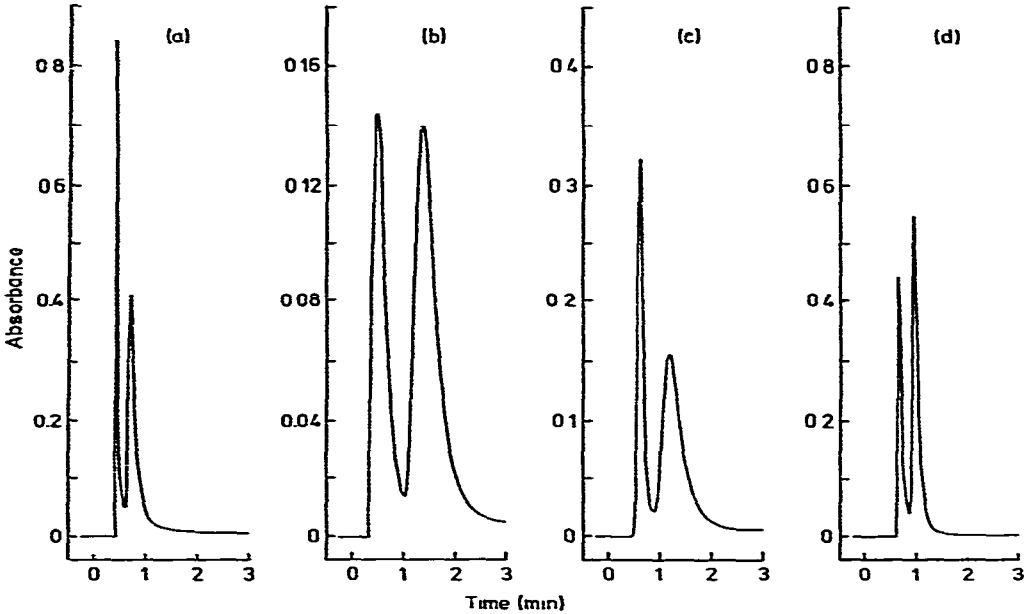

Fig. 3. Separation of 10 µl of a 5 mg/ml BSA–5 mg/ml Con A solution at 1.0 ml/min. The solution was injected at time zero onto columns of pore size 60 Å (a), 100 Å (b), 500 Å (c) and 4000 Å (d). The change from weak to strong mobile phase is not shown. The change was initiated prior to injection because of the holdup volume in the guard column. The Con A eluted with the strong mobile phase solvent front.

of flow-rate using the 500-Å column. The separation times were 2.4, 1.3 and 0.7 min at flow-rates of 0.5, 1.0 and 2.0 ml/min, respectively. Hence, the separation times were nearly inversely proportional to the flow-rate. This indicates that HPAC separations should not be severely degraded at high flow-rates. The reason for this is the relatively slow increase in H with flow-rate (see Figs. 1 and 2) even when the analyte peak is broadened by adsorption-desorption kinetics.

Capacity

In affinity chromatography, the adsorption capacity is the amount of analyte which will bind to the column. The capacity is often measured by saturating the column with analyte, washing the column, and measuring the amount of analyte after elution[24]. This was not possible to do here because the rather large dissociation constant of the Con A–affinity ligand complex caused the Con A to bleed from the column at an appreciable rate. Instead, a break-through curve[25] was obtained by continuously pumping a dilute (0.5 mg/ml) Con A solution through the column. The capacity was calculated from the 50% break-through volume[25] and is shown for each of the columns in Table VI, expressed as mg Con A/ml total column volume. The immobilized glucosamine concentrations and surface coverages are also shown.

Since the glucosamine surface coverages were all similar, the large differences in the glucosamine concentrations resulted from the decreased silica surface area as the pore size increased. The Con A capacity also reflected this trend, but partial exclusion from the 100-Å support decreased the available surface area and total exclusion from the 60-Å support greatly decreased the capacity.

TABLE VI

ADSORPTION CAPACITY FOR CON A

	Pore size			
	60 Å	*100 Å*	*500 Å*	*4000 Å*
Con A capacity* (mg/ml)	1 4	35	25	1 8
Glucosamine conc (μmole/ml)**	89	54	15	1 2
Glucosamine surface coverage (μmole/m²)***	0 28	0 50	0 70	0 38

* Determined using 0 5 mg/ml Con A The capacity was dependent on the Con A concentration used, indicating that the adsorption sites were not saturated

** Determined by the alkaline ferricyanide method[26] using N-acetyl-D-glucosamine as standard

*** Based on the manufacturer's (E Merck, Darmstadt, G F R.) estimates of surface area

It is interesting to note that the capacities of the 60-Å and 4000-Å columns were similar even though the Con A was totally excluded from the 60-Å column The capacities were much smaller than that of the 500-Å column. It is this higher capacity which made it more difficult to elute the Con A from the 500-Å column, as noted previously. It may be possible to improve separation time and sensitivity not only by altering the pore size (and thus the surface area), but also by decreasing the column size or ligand concentration since these variables also affect the capacity.

In previous HPAC studies, it has been observed that some of the analyte passed through the column unretained, and that the unretained fraction increased with flow-rate[2-4]. It was of interest to examine that problem here, particularly since the association rate constants of Con A for derivatives of α-D-mannopyranoside and α-D-glucopyranoside are reported to be unusually small[27,28]. Samples of 100 μg of Con A were injected at flow-rates up to 2 0 ml/min (u = 0 4 cm/sec) into the weak buffer In no case did any of the Con A elute unretained. However, Con A did elute isocratically ($k' \approx 4$) as a single broad peak from the 4000-Å column independent of the flow-rate This can be attributed to the large dissociation constant of the complex and the small amount of ligand in the column[29], and should not be a general problem since most analyte–ligand complexes have smaller dissociation constants.

CONCLUSIONS

To minimize HPAC band broadening and separation time, the following factors should be considered:

(1) Because of restricted diffusion, one should use supports having pore sizes which the sample components cannot penetrate (*e g* , 60-Å pores) or which are much larger than the sample components (*e.g.*, 4000-Å pores). Small solutes can easily penetrate the 60-Å pores, so problems could result if such molecules were even weakly retained on the large surface area inside the pores Totally non-porous supports should provide the highest efficiencies

(2) Small particles (10 μm or less) should always be used since plate height increases rapidly with particle diameter (see eqn 1)

(3) Since plate height does not increase as fast as flow-rate, reasonably high flow-rates (1–2 ml/min) can be used.

(4) To minimize band broadening of the analyte peak caused by adsorption–desorption kinetics, the strong mobile phase used for elution should reduce k' to as close to zero as possible. This is made easier if the capacity of the column is small. The sensitivity of the analysis will also improve.

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