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COMPARISON OF REVERSED-PHASE COLUMNS FOR THE SEPARATION OF TRYPTIC PEPTIDES BY GRADIENT ELUTION

CORRELATION OF EXPERIMENTAL RESULTS AND MODEL PREDICTION

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SUMMARY

A total of 10 reversed-phase columns obtained from different suppliers were evaluated for their ability to separate typical peptide mixtures: tryptic digests of cytochrome *c*, lysozyme and collagen. Each column was tested in a standard gradient elution using phosphate buffer (pH 2.85) as the A-solvent and acetonitrile as the B-solvent. Some differences in band spacing for the various peptides were observed from column to column. More important differences were observed in the ability of these various columns to provide narrow peaks and good resolution, as measured by column peak capacity. These differences in column peak capacity were related to differences in column dimensions and particle size; it also appeared that C_8 bonded phases were somewhat more efficient than were C_{18} phases. The recovery of cytochrome *c* digest from four of the columns was also assessed. Summed peak areas were proportional to sample size, as the latter was varied from 1 to 100 μg. The apparent recovery from each column did not vary by more than $\pm 5\%$. From this it was concluded that these columns gave essentially quantitative recovery for this particular sample. Limited data on column life were obtained for some of these columns.

INTRODUCTION

The separation of peptides and small proteins by reversed-phase high-performance liquid chromatography (HPLC) is today a widely used technique[1–8]. In response to this need, column manufacturers have made available a large number of different columns which can be used for these peptide separations. These columns differ in column dimensions, particle size, pore diameter and the kind of bonded phase. The question then arises, what combination of column characteristics is generally optimal for these peptide separations? Various groups have attempted to answer this question

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on the basis of either empirical or theoretical arguments (or both)[4–8]. Generally it is believed that larger pores should give better separation for small sample sizes, but large-pore columns will more quickly become overloaded because of their smaller surface area. Likewise, several studies have suggested that separation and recovery are generally poorer on long, alkyl-chain phases, particularly C_{18}. Theory and experiment agree in suggesting that smaller particles are able to effect faster, higher-resolution separations, particularly as the molecular weight of the sample increases.

We have attempted to provide further insight into what makes a "good" column for these peptide separations. We studied a total of 10 commercial columns, varying in particle size, pore diameter and in the kind of bonded phase (C_8 *vs.* C_{18}). Standard gradient elutions were carried out with each column, using various tryptic digests (cytochrome *c*, lysozyme, collagen) as sample. We examined differences in relative band spacing, column efficiency (as measured by band width or peak capacity) and sample recovery. Differences in peak capacity were further analyzed in terms of a model for peptide separations by reversed-phase gradient elution[8].

EXPERIMENTAL

Columns

Columns were obtained from the manufacturers as follows: Du Pont Zorbax® C_8 columns, 8 × 0.62 cm I.D., 4 or 5 μm particle size, 150 Å pore size, 3.2 μmoles bonded phase per m^2 (similar 5-μm columns are available as Bioseries PEP-RP1); Du Pont Zorbax C_{18} columns, 25 × 0.46 cm I.D., 5 μm particle size, 150 Å pore size, 2.6 μmoles bonded phase per m^2. Three each of the latter columns were the gift of E.I. du Pont de Nemours & Co. (Biomedical Products Dept., E.I. du Pont de Nemours & Co., Wilmington, DE, U.S.A.); Waters μBondapak® column, 30 × 0.39 cm I.D., 10 μm particle size, 125 Å pore size, 3.0 μmoles bonded phase per m^2; Waters NovaPak® column, 15 × 0.46 cm I.D., 4 μm particle size, 90 Å pore size, 3.0 μmoles bonded phase per m^2. These columns were the gifts of Waters Assoc. (Milford, MA, U.S.A.); Altex Ultrasphere® ODS C_{18} column, 25 × 0.46 cm I.D., 5 μm particle size, 80 Å pore size, 2.9 μmoles bonded phase per m^2, purchased from Beckman Instruments (Beckman Instruments, Altex Scientific Operations, Berkeley, CA, U.S.A.); Perkin-Elmer 3 × 3 C_{18} column, 3 × 0.4 cm I.D., 3 μm particle size, 100 Å pore size, a gift from Perkin-Elmer (Norwalk, CT, U.S.A.).

Chromatography

Chromatography was performed with a Waters Assoc. gradient liquid chromatography system including two M 6000A pumps, system controller, 440 ultraviolet detector equipped with an extended wavelength module for detection at 214 nm; a Shimadzu RF 530 fluorometer with low-pressure xenon lamp, excitation 295 nm, emission 395 nm (Shimadzu, Columbia, MD, U.S.A.); data module and DYSC integration system in concert with an Apple 2+ computer (Dynamic Solutions, Pasadena, CA, U.S.A.).

HPLC-grade reagents were used throughout the study. All glassware used was cleaned in an acid bath prior to use. New lots of Fisher or Burdick & Jackson acetonitrile and methanol were assayed and selected for low ultraviolet absorbance and fluorescence (Fisher Scientific, Fair Lawn, NJ, U.S.A.; Burdick & Jackson Labs.,

Muskegon, MI, U.S.A.). All mobile phases were filtered through a fine, sintered glass filter and degassed prior to use; they then were sparged with helium and maintained under continuous helium pressure (3 lb.) during chromatography. Effluent from the acetonitrile pump first passed through an in-line column of alumina (Aluminar, Mallinckrodt, Paris, KY, U.S.A.) and charcoal (Nucleotide desalting grade, Aldrich, Milwaukee, WI, U.S.A.) before flowing to the mixing chamber. Effluent from the aqueous pump passed through an in-line column of Chromosorb 101 (Supelco, Bellefonte, PA, U.S.A.) and then charcoal, prior to flowing to the mixing chamber[9].

The gradient was linear from 100% 0.05 *M* phosphate pH 2.85 (A) in 114 min to 28% acetonitrile (B), followed by 34 min of a linear gradient from 28% B to 62% B with a 20 min hold at 62% B then a 6-min re-equilibration in 100% A. Almost all peaks were eluted during the 114-min gradient. The flow-rate was 1 ml/min at 25°C (modified from O'Hare and Nice[10]). A Gilson Model 201 fraction collector (Gilson Medical Electronics, Middletown, WI, U.S.A.) was used for collection of peptides for amino acid analysis. After operation, columns were pumped with 30 ml of methanol–water (10:90) and stored in methanol–water (10:90).

Sample preparation

Trypsin digests of cytochrome *c* (horse heart) and lysozyme (egg white lysozyme) (Sigma No. L-6876) were prepared according to the method of Canfield and Anfinsen[11]. Lysozyme was reduced and alkylated. For preparation of trypsin digests, lysozyme or cytochrome *c* were dissolved in 0.02 *M* NH_4HCO_3 pH 8.0 at 10 mg/ml. The solution was made 1% (w/w) with respect to trypsin in 0.01 *N* HCl [L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) modified trypsin, Calbiochem, San Diego, CA, U.S.A.]. The solution was incubated with stirring at 37°C for 10 h. After enzyme hydrolysis, the solution was heated to 60°C for 20 min, cooled and half the initial trypsin concentration added, then the solution was incubated for an additional 10 h at 37°C. The hydrolyzate was sedimented at 10000 *g* for 10 min, the supernatant fluid was collected, lyophilized, and stored at −20°C. Sample portions were reconstituted in 0.05 *M* phosphate buffer pH 2.85.

Trypsin hydrolyzates of nonmineralized and mineralized Type I bone collagen were prepared as described with the following modifications: 100 mg bone powder were treated with 1 ml 0.5 mg/ml RNase (R-5000), DNase (D-4638) (Sigma, St. Louis, MO, U.S.A.) for 30 min at 37°C, washed, then treated twice with 0.5% TPCK trypsin for 30 min per treatment to remove noncollagen proteins[12].

Calculation of peak capacity and peak asymmetry values

Peak capacity, PC, was calculated according to the equation[13]

$$\mathrm{PC} = \frac{t_g - t_0}{4\sigma_t} = \frac{t_g - t_0}{1.699 \text{ width at } \frac{1}{2} \text{ height}}$$

where PC = Peak capacity (resolution $R_s = 1$), t_g = elution time in seconds of the last peak (at mid-peak), t_0 = elution time in seconds of an unretained solute and $4\sigma_t$ = width of the peak at baseline (4σ).

Five separate peaks were selected from the beginning, middle and end of each

TABLE I

COLUMN PERFORMANCE DATA FOR GRADIENT SEPARATION OF LYSOZYME AND CYTOCHROME *c* TRYPSIN DIGESTS

Column	*Alkyl group*	*Column size (cm)*	*Particle diameter (μm)*	*Pore diameter (nm)*	*Lysozyme data*				*Cytochrome c peak capacity*	
					*As.**	*Peak capacity*				
						Exptl.	*Calc.***	*Ratio****	*Exptl.*	*Ratio*§
μBondapak	C_{18}	30 × 0.39	10	12.5	1.12	128 ± 2§§	103	1.24	143 ± 6	1.12
NovaPak	C_{18}	15 × 0.46	4	9	1.15	163 ± 7	166	0.98	181 ± 6	1.11
P/E 3 × 3	C_{18}	3.3 × 0.46	3	10	1.33	103 ± 3	78	1.32	138 ± 2	1.33
Altex	C_{18}	25 × 0.46	5	10	0.96	193 ± 1	169	1.14		
Altex (used)§§§	C_{18}	25 × 0.46	5	10	2.58	59 ± 1	169	(0.35)	110 ± 4	(1.88)
Zorbax A†	C_8	8 × 0.62	4	15	1.00	178 ± 3	125	1.42	211 ± 3	1.19
Zorbax A (used)					1.06	172 ± 3		(1.38)	214 ± 7	1.24
Zorbax B					1.02	179 ± 8		1.43	219 ± 2	1.22
Zorbax B (used)					1.10	174 ± 7		(1.55)	204 ± 5	1.17
Zorbax C	C_8	8 × 0.46	5	15	0.90	160 ± 4	112	1.43	203 ± 7	1.26
Zorbax C (used)					1.15	106 ± 1		(0.95)	117 ± 2	1.10
Zorbax D	C_{18}	25 × 0.46	5	15	1.15	176 ± 7	169	1.04	198 ± 13	1.12
Zorbax E					0.98	166 ± 3		0.98	188 ± 9	1.13
Zorbax F					0.99	167 ± 2		0.99	192 ± 10	1.15

* Peak asymmetry factor.

** Calculated according to ref. 8.

*** Ratio (exptl. peak capacity)/(calc. peak capacity).

§ Ratio (exptl. peak capacity for cytochrome *c*)/(exptl. peak capacity for lysozyme).

§§ Variation reported as 1 std. dev. for 3 replicate runs.

§§§ Altex column after 11 000 column volumes of mobile phase (over one year of use).

† Columns of the same type include (A, B) and (D, E, F); for column histories, see Table II.

chromatogram (up to 114 min, 0.28% B, linear) and peak capacities were calculated and averaged. Determinations were performed in triplicate for each column.

Peak asymmetry values were calculated as reported[13].

Peptide recovery experiment

A series of injections of cytochrome *c* trypsin digest were made into each column. The columns, after storage in methanol–water (10:90), were equilibrated in 0.05 *M* phosphate buffer pH 2.85 until the baseline was stable. The first sample of each group was a buffer blank, followed by single injections in series of 1, 2.5, 5, 10, 20, 35, 50, 60 or 65, 70 or 75, 80, 90, 100 then 5 μg cytochrome *c* tryptic peptides from a 1 mg/ml stock solution. The lower masses (1, 2.5, 5 μg) were diluted and injected in 10 μl. Total area detected was plotted as a function of amount injected.

Chromatography, isolation and identification of tryptic peptides of cytochrome c

Tryptic peptide samples were injected in triplicate onto the various test columns and eluted as described, in the Experimental section, above. For cytochrome *c* peptides, 1 mg of digest was applied in 130 μl and separated in the standard gradient. Individual peptides were collected, lyophilized, a portion rechromatographed to test purity, then another portion hydrolyzed in 6 *N* HCl for 24 h, dried, then derivatized with phenylisothiocyanate, and the phenylthiocarbamyl derivatives separated at 38°C in a 12-min gradient of sodium acetate and acetonitrile (Waters Assoc. Pico-Tag amino acid analysis system).

RESULTS AND DISCUSSION

Data on the 10 columns studied by us are summarized in Table I; additional data on column life are given in Table II. The columns in Table I include 7 different column types, differing in source (supplier) and column characteristics. In several cases, different columns of the same type were investigated, in order to determine column-to-column variability. The results in Table I suggest that columns of the same type generally perform in similar fashion. Data for three runs on each column were used to obtain the average figures summarized in Table I. Chromatograms for the separation of the cytochrome *c* digest on each column type are shown in Figs. 1–6 for direct comparisons of column performance. Figs. 7 and 8 show similar separations of the lysozyme and collagen digests. The complexity of the latter sample illustrates the high resolving power of this particular separation; about 86 separate peaks can be recognized in the chromatogram.

Column resolving power

It is not immediately apparent from an examination of Figs. 1–6 which column is "best" for this particular sample, or for peptide separations in general. Furthermore, the column types represented here differ in many respects, and it is difficult to recognize which aspect of column design contributes most significantly to column "goodness". Fortunately, a general model is available for predicting column performance as a function of column configuration and conditions of separation[8]. We will use this model in analyzing the data in Table I.

Resolution or separation is a function of two factors: (1) the peak capacity of

TABLE II

COLUMN PERFORMANCE *VS.* COLUMN LIFE (DATA OF TABLE I)

Actual running time and use for carrying out separations are similar to that of the standard gradient run described in Experimental.

Column	*Mobile phase (column volumes) passed through column*	*Samples injected*	*Elapsed time (months)*	*Peak capacity**		*Asymmetry* factor*	
				Initial	*Final*	*Initial*	*Final*
Altex C_{18}	11000	180	12	193	59	0.96	2.58
Zorbax C	15642	148	12	160	106	0.90	1.15
Zorbax A	2647	25	1	172	179	1.00	1.06

* Lysozyme digest.

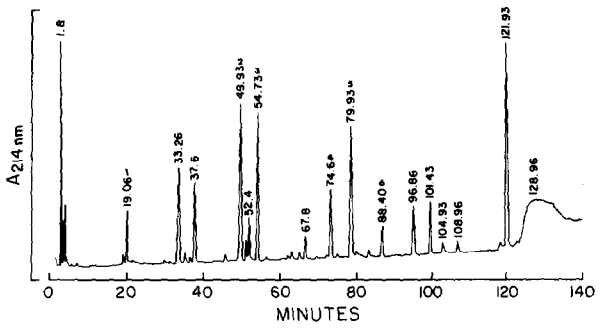

Fig. 1. Separation of cytochrome *c* tryptic digest on a Waters NovaPak column with standard gradient protocol (see Experimental). Nonhydrolyzed cytochrome *c* is eluted at 121.9 min. The following peptides were collected and sequenced for identification: 19.1 min (NH_2GDVEK); 49.9 min (YIPGTK); 54.7 min (TGQAPGFTYTDANK); 74.6 min (CAQCHTVEK); 79.9 min (TGPNLHGLFGR); 88.4 (ADLIAYLK).

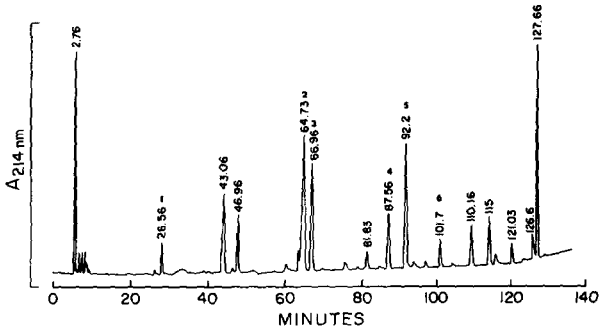

Fig. 2. Separation of cytochrome *c* tryptic digest on a Waters μBondapak column. Conditions as in Fig. 1.

the column, equal to the number of peaks that could be separated with resolution $R_s = 1$, if all bands were evenly distributed within the chromatogram, and (2) the relative band-spacing of different peaks in an actual separation. For the general case, we cannot anticipate how a particular column will affect band-spacing, but a higher

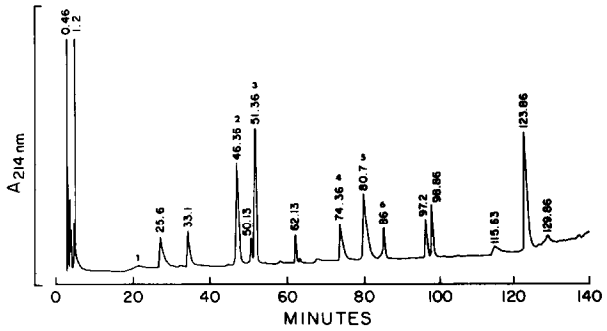

Fig. 3. Separations of cytochrome *c* tryptic digest on a Perkin-Elmer 3 × 3 column. Conditions as in Fig. 1.

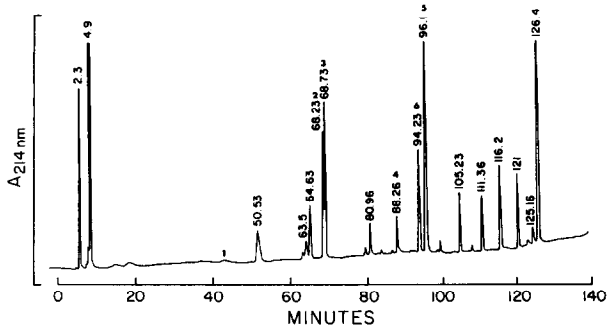

Fig. 4. Separation of cytochrome *c* tryptic digest on a Beckman-Altex Ultrasphere C_{18} column. Conditions as in Fig. 1.

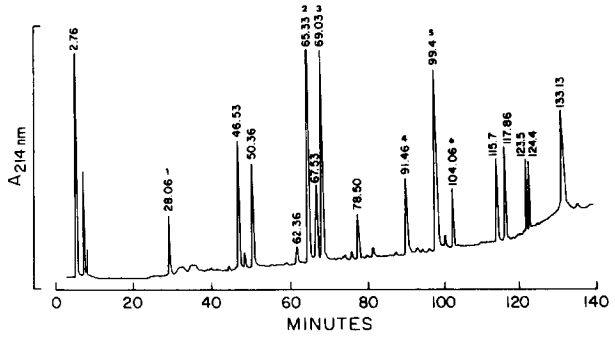

Fig. 5. Separation of cytochrome *c* tryptic digest on a Zorbax C_{18} column. Conditions as in Fig. 1.

peak capacity will be generally advantageous for all samples. Likewise, bands which do not tail (have asymmetry factors near one), will be preferred.

Peak capacity and band asymmetry values. If we consider only data for the 10 new columns in Table I, we see that peak capacity values vary from 103 to 193 for the lysozyme digest, and from 138 to 219 for the cytochrome *c* digest; *i.e.*, by a factor of 1.6 to 1.8. If we exclude the P/E 3 × 3 column in Table I, the asymmetry factors for the remaining columns average 1.03 ± 0.05; *i.e.*, there is not much variation. The

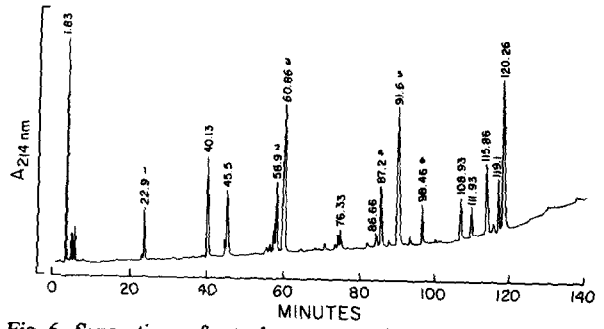

Fig. 6. Separations of cytochrome *c* tryptic digest on a Zorbax 5-μm C_8 column (commercially available as the Du Pont Bioseries PEP-RP1). Conditions as in Fig. 1.

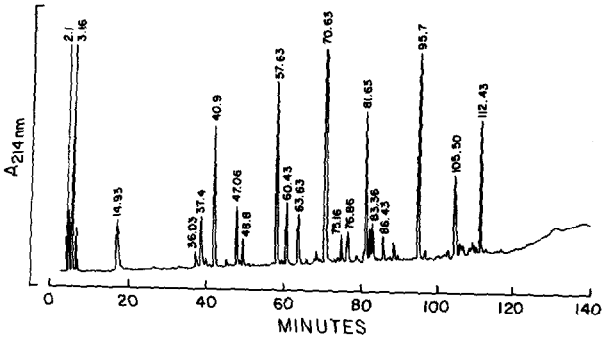

Fig. 7. Separation of lysozyme tryptic digest on a Zorbax 5-μm C_8 column. Conditions as in Fig. 1.

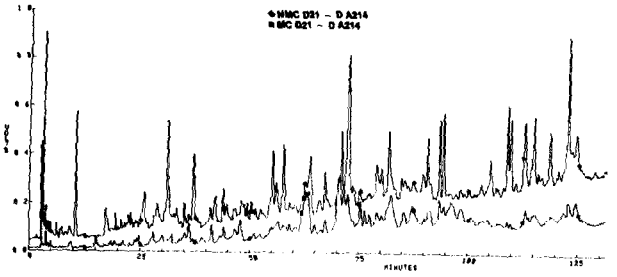

Fig. 8. Separation of collagen tryptic digest on a Zorbax 5-μm C_8 column. Conditions as in Fig. 1. NMC = nonmineralized collagen tryptic peptides. MC = mineralized collagen tryptic peptides from 21-day-old vitamin D-deficient rat pup bone. The ordinate is absorbance at 214 nm.

Perkin-Elmer 3 × 3 column, because of its small column volume and small particles, is subject to extra-column effects (band tailing) in the conventional HPLC system used for these experiments.

The peak capacities for the cytochrome *c* experiments closely parallel those for lysozyme. Thus, the average ratio (peak capacity lysozyme)/(peak capacity cytochrome *c*) for the different columns equals 1.18 ± 0.07; *i.e.*, there is little variation

from column to column. Therefore, we can analyze the lysozyme peak capacity values, knowing that our conclusions will apply also to the cytochrome *c* data.

The calculated peak capacities for lysozyme (Table I) are based on a particular column-packing type: a C_8 15-nm pore particle[8]. However, we have calculated peak capacities for each column, ignoring differences in alkyl chain length and pore size. These calculated peak capacities take into account differences in column length and diameter and in particle diameter. Note first that the range of calculated peak capacities in Table I is fairly wide, 103–169; *i.e.* column dimensions and particle size contribute significantly to differences in experimental peak capacity values. These contributions to column resolving power have been analyzed in detail elsewhere[8]. We can summarize them by stating that, whereas the column diameter is relatively unimportant, the column length can be important, and the column particle diameter is very important.

These effects can be seen in the calculated values of peak capacity in Table I. Thus the Perkin-Elmer 3 × 3 column has a rather low peak capacity value because of its short length. Under the conditions of separation, the reduced velocity of the mobile phase is about 3, which corresponds approximately to the optimum velocity for minimum plate height. Under these circumstances, the performance of the column is mainly limited by its length. Similarly, the rather low peak capacity of the μBondapak column is due to its large particle size (10 μm *vs.* 3–5 μm for the remaining columns). However, it should be stressed that optimum column dimensions and particle size (resulting in maximum peak capacity), depend upon sample molecular weight and other experimental conditions (gradient time, flow-rate, etc.) as discussed in ref. 8.

Consider next the ratio (experimental peak capacity)/(calculated peak capacity), equal to 1.20 ± 0.14, for the columns under discussion (new columns in Table I). Note first that this value is close to 1, which corresponds to an exact agreement between peak capacities derived experimentally and those calculated on the basis of a theoretical model[8]. Note also that the variation in this ratio among the 10 new columns of Table I is rather small, but is significant when compared to the experimental variability of these peak capacity values from chromatogram to chromatogram (±3%), or from column to column (±4%). This suggests that other factors (pore diameter, alkyl chain length) may also be significant in contributing to column performance. We can group these ratios of experimental to calculated peak capacity values as follows:

Pore size	*C_8 packing*	*C_{18} packing*
<11 nm	–	1.06 + 0.09
>11 nm	1.42 + 0.01	1.06 + 0.11

These results clearly suggest that C_8 packings are more effective (larger peak capacities) than C_{18} packings in the separation of peptide mixtures. There seems to be no obvious advantage of larger pores (12–15 nm) over smaller pores (9–10 nm). The latter observation may be due to the low average molecular weight of these samples (about 1000 daltons) and to the particular experimental conditions employed.

Peak capacity vs. conditions. As a further check on the model of ref. 8, as used here to analyze contribution to peak capacity, additional measurements were made as a function of varying gradient time and mobile-phase flow-rate. These peak capacity data, summarized in Table III, were obtained with the Du Pont 8 × 0.45 cm, 5-nm pore C_8 column (the same column with which the model was developed; currently available as the Du Pont Bioseries PEP-RP1). Calculated band-width values correlated quite closely with experimental values (ratio = 1.35 ± 0.06), but were consistently greater by 35%. At present, we believe that this difference may be related to the use of a phosphate buffer as the mobile phase in the current study, compared to trifluoroacetic acid used in the model development[8]. However, it is clear that the model accurately accounts for relative changes in band width and peak capacity as experimental conditions are changed. The model should therefore be applicable in correcting for the effects of column dimensions and particle size on peak capacity (as used in Table I).

TABLE III

BAND-WIDTH MEASUREMENTS FOR THE DU PONT 8 × 0.46 cm 15-nm PORE, C_8 COLUMN AS A FUNCTION OF GRADIENT TIME AND FLOW-RATE

Band-width measurements are in seconds (1 S.D.) for peaks of lysozyme tryptic peptides chromatographed with a phosphate–acetonitrile system as in Experimental.

Gradient time (min)	*Flow-rate (ml/min)*								
	0.5			*1*			*2.5*		
	Exptl.	*Calc.*	*Ratio*	*Exptl.*	*Calc.*	*Ratio*	*Exptl.*	*Calc.*	*Ratio*
30	4.1	5.8	1.41	3.6	5.1	1.42	3.3	4.7	1.42
60	6.9	8.4	1.22	5.6	7.8	1.39	5.6	7.9	1.38
120	11.0	13.7	1.25	9.0	13.4	1.30	10.2	14.0	1.37

Variations in sample retention among different columns. The chromatograms of Figs. 1–6 for the separation of the cytochrome *c* tryptic digest on different column types show changes in both absolute and relative retention of individual bands. Some of these retention differences are due to differences in column volume. Thus, it has been shown for the gradient elution separation of peptides[14,15] that systematic changes in relative band position result as the volume, V, of the column is increased (or if either flow-rate or gradient time are increased). The early part of the chromatograms in Figs. 1–6 appears to be correlated with column volume, in that smaller columns (Perkin-Elmer 3 × 3) have lower retention times and larger columns (25 × 0.46 cm) have greater retention times for the same bands. However, other differences apart from these also exist and probably reflect column-to-column variability in the retention of specific peptides (see discussion in ref. 3, pp. 101–103). For a given column, there is a linear relationship (r = 0.98 for peptides 1, 2, 3, 5, 6) for elution time *versus* the sum of the retention coefficients for the constituent amino acids, as proposed by Meek (ref. 16, Table 4).

TABLE IV

RETENTION TIMES, SEQUENCE, POLARITY, CHARGE AND RETENTION COEFFICIENTS FOR CYTOCHROME *c* TRYPTIC PEPTIDES

1 and 2 are primary and secondary retention times, respectively, for chromatography in a NovaPak C_{18} column with a phosphate–acetonitrile gradient. Polar, nonpolar, + and − refer to the groups of amino acids in these selected tryptic peptides of cytochrome *c*. R_F is the sum of the retention coefficients for the constituent amino acids. There is a linear relationship between summed R_F and retention time, except for the cysteine-containing peptide (after Meek[14]).

Peak No.	*1*	*2*	*Sequence*	*Polar*	*Nonpolar*	+	−	R_F	*Residues*
1	20.7	18.7	H_2N-GDVEK	1	1	1	2	−4.5	5
2	50.4	50.2	YIPGTK	3	2	1	0	25.8	6
3	55.4	55.84	TGQAPGFTYTDANK	8	4	1	0	28.6	14
4	74.6	76.3	CAQCHTVEK	4	2	2	1	−4.6	9
5	79.8	80.9	TGPNLHGLFGR	5	3	2	0	38.2	10
6	88.8	88.9	ADLIAYLK	1	5	1	1	35.0	8

Column stability

Our laboratory has been analyzing peptide digests, such as those in Figs. 1–8, for more than four years, and in that time many hundreds of samples have been chromatographed. During this period no unusual problems have been encountered with column stability or lifetime, and we are accustomed to using a column for a year or more. Some limited observations on column stability are presented in Table II.

Column life is probably a function of (1) the number of samples separated, (2) the total volume of mobile phase that has passed through the column, and (3) the length of time during which the column is used. Additionally, (4) the mobile phase composition and (5) temperature, as well as (6) storage conditions of the column between analyses are known to play an important role in determining column life. The data in Table II indicate that as the column ages, peak capacity decreases and band asymmetry increases. However, the effect on different samples can vary, as seen in Table I for the Altex column used; note the lysozyme sample (peak capacity = 59) *vs.* the cytochrome *c* sample (peak capacity = 110).

The present gradient protocol consumes a rather large volume of mobile phase per analysis (typically 60–100 column volumes). Under these conditions, the major contributor to column "death" may be exposure of the column to the flow of mobile phase. This could account for the rather small number of samples that can be injected (*ca.* 200) before column "death", assuming that the data of Table II are representative. Thus, for other HPLC separations requiring less time and volume, good columns should last for 1000–2000 sample injections.

Sample recovery

Recovery of separated peptides from the cytochrome *c* samples was studied over the range 1–100 μg sample injected, for four columns from Table I. The protocol involved injections from low to high amounts, followed by a final 5-μg injection that duplicated an initial 5-μg sample. The initial and final 5-μg injections gave total

(summed) area counts for the chromatograms that were equal within experimental error for all columns. All plots of summed area *vs.* sample amount (1–100 μg) were linear and transected the origin (Fig. 9), with relative slopes for the four columns as follows: Altex (1.00), Zorbax C_{18} (1.03), Zorbax C_8 (1.03), Novapak (1.10). Plots of band area *vs.* sample amount for individual peptides gave similar linear plots. When these data were plotted (summed areas) for a lower range of sample amount (1–20 μg), the slopes were identical within experimental error for all four columns. We conclude from these observations that sample recovery is quantitative within experimental error for all four columns, as has been noted by others[16,17].

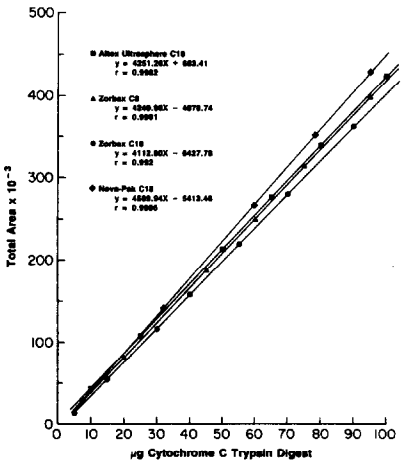

Fig. 9. Plot of total band area for chromatogram *vs.* sample size: linear regression lines of peptide mass *vs.* area for reversed-phase columns. Standard gradient conditions as in Experimental.

CONCLUSIONS

We studied the performance of 10 commercial reversed-phase columns with a standard gradient elution separation of various protein digests (lysozyme, cytochrome *c* and collagen). These columns differed in column dimensions, particle size, pore diameter and C_8 or C_{18} bonded phases. They showed differences in resolving power, as measured by peak capacity. Peak capacity values for the different columns were compared with a quantitative model in an effort to elucidate the factors that determine column resolving power[8]. We concluded that column length and particle size contributed to the peak capacity of these columns, and that C_8 columns were significantly more efficient (had larger peak capacities) than were C_{18} columns, other factors being equal. When differences in column dimensions, particle size and alkyl chain length were taken into account, relative peak capacities could be predicted with an accuracy of $\pm$ 8%. However, other studies indicate that relative peak capacity can vary more widely as sample molecular weight or gradient conditions are changed[8]. In related studies, for one column where gradient conditions were changed (gradient time and flow-rate), the theoretical model accurately predicted changes in relative band width or peak capacity for the different chromatograms[8]. Peak capa-

cities for both the lysozyme and cytochrome *c* tryptic digests closely paralleled each other, suggesting that our findings will be general for other protein digest samples.

Changes in both absolute and relative retention for various bands within the chromatograms were noted among the different columns. Some of these changes in retention appeared to be due to the different column sizes (dead volume, V_m), but in most cases they appeared to reflect differences in the bonded phase (secondary interactions with silanols, etc.).

Some data were obtained on column life or stability. We found that columns typically lasted for about 200 sample injections in our system, a total mobile-phase flow through the column of 10000–15000 column volumes, and/or up to a year.

Studies were also carried out on the recovery of 1–100 μg samples of cytochrome *c* digest injected into 4 of these columns. Analysis of these data suggests that all 4 columns give quantitative recoveries within experimental error.

REFERENCES

1 M. T. W. Hearn, F. E. Regnier and C. T. Wehr, *Amer. Lab.*, 14 (1982) 18.
2 W. S. Hancock and J. T. Sparrow, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography: Advances and Perspectives*, Vol. 3, Academic Press, New York, 1983, p. 49.
3 M. T. W. Hearn, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography: Advances and Perspectives*, Vol. 3, Academic Press, New York, 1983, p. 87.
4 J. M. Di Bussolo, *Amer. Biotechnology Lab.*, June 1984, p. 20.
5 E. C. Nice, M. W. Capp, N. Cooke and M. J. O'Hare, *J. Chromatogr.*, 218 (1981) 569.
6 J. D. Pearson and F. E. Regnier, *J. Liquid Chromatogr.*, 6 (1983) 497.
7 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 296 (1984) 61.
8 M. A. Stadalius, M. A. Quarry and L. R. Snyder, *J. Chromatogr.*, 327 (1985) in press.
9 V. Berry, *J. Chromatogr.*, 236 (1982) 279.
10 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.
11 R. E. Canfield and C. B. Anfinsen, *J. Biol. Chem.*, 238 (1963) 2684.
12 A. J. Banes, M. Yamauchi and G. L. Mechanic, *Biochem. Biophys. Res. Commun.*, 113 (1983) 975.
13 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979.
14 M. A. Stadalius, H. S. Gold and L. R. Snyder, *J. Chromatogr.*, 296 (1984) 31.
15 J. L. Glajch, M. A. Quarry, J. F. Vasta and L. R. Snyder, *Anal. Chem.*, in press.
16 J. L. Meek, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1632.
17 M. D. Waterfield and G. T. Scrace, in G. L. Hawk (Editor), *Biological/Biomedical Applications of Liquid Chromatography III*, Marcel Dekker, New York, 1981, p. 135.