Improved reversed-phase high performance liquid chromatography columns for biopharmaceutical analysis [☆]

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Abstract

Reversed-phase high performance liquid chromatography continues to grow in importance for the analysis of peptides and proteins in biomolecular and pharmaceutical research. The mobile-phase conditions for separation of proteins and peptides are essentially fixed. Most separations of these solutes are conducted with shallow gradients using aqueous buffers modified with acetonitrile. Therefore, changing selectivity in peptide and protein separations is often best accomplished by a change in bonded-phase chemistry of the column (e.g. by using CN- or C3-bonded phases rather than C8 or C18). In general, however, short-chain bonded phases are more unstable and irreproducible than bonded phases having longer chains, due to increased susceptibility to hydrolysis as hydrophobicity of the bonded phase decreases. These problems are minimized using bonded phases that are protected through use of sterically bulky side-groups (StableBond Technology). This paper describes a series of comparisons between traditional polymeric bonded-phase silica columns and sterically protected, highly purified, silica stationary phases. These studies compare lot-to-lot reproducibility, stability of the bonded phase, selectivity effects between bonded phases, and operational advantages that can be obtained by high temperature operation using a series of stable bonded phases.

Keywords: Reversed-phase HPLC; Chromatographic reproducibility; Chromatographic stability; Bonded-phase selectivity; Temperature selectivity; Proteins; Peptides

1. Introduction

Contemporary reversed-phase high performance liquid chromatography (HPLC) of peptides and proteins is best carried out using gradients of aqueous organic mixtures containing 0.1% TFA. This limits the chromatographer's ability to optimize separations by mobile-phase changes. Monomerically bonded, sterically protected, bonded phases on low acidity, high purity silica have been available for a number of years. This silica is produced from start to finish to meet strict standards; it is fully hydroxylated, has low acidity, and is of 99.995% purity. The bonded phases are protected through bulky side groups,

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reducing hydrolysis of the siloxane bond at low pH. These stationary-phase properties yield columns that are reproducible and extremely stable at low pH and high temperature [1–4]. This increase in stability applies also to short-chain bonded phases that have traditionally been significantly less stable than long-chain bonded phases, leading to instability and reduced ability in obtaining robust methods [5,6]. New tools for polypeptide separations are available through an expanding line of columns based upon wide-pore (300 Å) packing materials with these same characteristics.

Changes of bonded phase and temperature to effect separation of small molecules and peptides has been demonstrated [7]. More recently, these techniques were used on 300 Å SB columns to demonstrate use of different bonded phases to obtain separations [8] and use of temperature to optimize separations [9,10]. In the current study, purified peptide and protein samples were used to methodically explore practical advantages of high reproducibility, extreme low-pH stability, change of selectivity with bonded-phase type, and change of selectivity with temperature.

Results in this paper show that changes in selectivity by varying the bonded phase and changing the operating temperature are viable alternatives to modifications of the mobile phase. Examples of significant change in resolution effected by reduced peak-width and/or molecule-specific changes in retention are shown. As important factors in method development and validation, the data presented also show the unprecedented lot-to-lot reproducibility and long-term low-pH stability of these column packings.

2. Experimental

2.1. Instrumentation

HPLC was performed using Ti-Series HP-1050 or HP-1090 liquid chromatographs (Hewlett-Packard, Valley Forge, PA, USA) equipped with quaternary pumps, variable injection-volume autosamplers and variable-wavelength detectors. Both HP systems were equipped with built-in

column heaters controlled from the front panel of the main unit. Data were collected with an IBM-compatible 80486 computer running ChromPerfect Direct 4I (version 1.1) or ChromPerfect for Nelson chromatography software with a DT2804 data-acquisition card (Justic Innovations, Mountain View, CA, USA).

2.2. Columns

The StableBond® columns used were Zorbax® 300SB-CN, -C3, -C8, and -C18 (MAC MOD Analytical, Chadds Ford, PA, USA). These columns (4.6 i.d. × 150 mm) contained 5 μm particles with a pore diameter of 300 Å. Additional column types used in the stability study included a Zorbax SB-C18 (80 Å) and Zorbax Rx-C18 (MAC MOD Analytical, Chadds Ford, PA, USA), and a Vydac C18 and C4 (4.6 mm × 150 mm) (The Separations Group, Hesperia, CA, USA). A Hypersil ODS column (4.6 mm × 75 mm) was also used (Keystone Scientific, Bellefonte, PA, USA).

2.3. Mobile phases

Mobile phases were prepared with deionized, prefiltered water (IONPURE, mixed bed ultra) that was additionally filtered with a hydrophilic 0.22 μm filter (Millipore, Bedford, MA, USA) before mixing. Mobile phases were prepared with HPLC-grade acetonitrile (ACN; EM Science, Gibbstown, NJ, USA) and trifluoroacetic acid (TFA; Pierce Chemical, Rockford, IL, USA) stored below room temperature. HPLC-grade methanol was purchased from EM Science.

2.4. Column reproducibility

To test the Zorbax 300 SB-C18 column, a mixture of 10-residue synthetic peptides (Alberta Peptides Institute, Department of Biochemistry, University of Alberta, Edmonton, Alta., Canada) was diluted to 0.6 mg ml⁻¹ in water–TFA (99.9:0.1, v/v) and injected (10 μl). The decapeptides increase slightly in hydrophobicity from P1 to P5. The mobile phase used was water–TFA (99.9:0.1, v/v) for solvent A, and ACN–TFA (99.91:0.09, v/v) for solvent B. The gradient was

4–32% B in 30 min. Other conditions were as indicated in the individual figures.

To test the Zorbax 300 SB-C3 column, protein standards (10 mg ml^{-1}) were first dissolved in guanidine–HCl (pH 7.0, 6 M) to denature the structure. An eight-component sample (0.05–0.250 mg ml^{-1}) was then prepared in solvent A (above) from individual 10 mg ml^{-1} protein and peptide standards. Bovine insulin contained, in addition, TFA (6% by volume) to increase solubility. The mobile phase for the eight-component sample was water–ACN–TFA (95:5:0.10, v/v/v) for solvent A and water–ACN–TFA (5:95:0.085, v/v/v) for solvent B. The gradient was 15–53% B in 20 min. Other conditions are as shown in individual Figures and Tables. Columns used in this study were Zorbax 300SB-C3 (4.6 i.d. × 150 mm) prepared from five different lots. Each column was conditioned with solvent A–solvent B (15:85, v/v) for 50 column volumes, (60:40, v/v) A–B for 5 column volumes, and was then equilibrated before use with 12 column volumes (for a 4.6 i.d. × 150 mm column, one column volume = 1.6 ml).

2.5. Stability

To observe the stability of the sterically protected Zorbax 300SB-C18 and 300SB-C3 columns, low pH, high-temperature environments were used. The Zorbax 300SB-C18 and two commercially available C18 columns were continuously purged with methanol–water–TFA (50:49:1.0, v/v/v), pH* 0.8 at 90 °C. Toluene (used as a neutral test probe) was injected to observe any loss in retention due to loss of bonded phase. The 300SB-C3 column was tested by continuously repeating gradients of 0–100% B using a mobile phase of H$_2$O–TFA (99.50:0.50, v/v) for solvent A and ACN–TFA (99.5:0.5, v/v). Phenylheptane was injected and chromatographed isocratically using a mobile phase of ACN–H$_2$O–TFA (70:29.9:0.1, v/v/v) to observe any degradation of the bonded phase, as detected by loss in retention.

2.6. Bonded phase and temperature selectivity

Protein samples were prepared from the same

10 mg ml^{-1} stock as the eight-component sample above. To detect any change in protein retention time or peak shape due to an increase in column temperature (ambient to 60 °C) or a change in the bonded phase, two samples containing 3–4 closely eluting (k' difference of 2 or less) proteins or peptides were prepared in guanidine HCl (pH 7.0, 6 M). The first sample contained 0.20 mg ml^{-1} of each protein and the second sample was spiked by increasing one or two protein concentrations to 0.40–0.6 mg ml^{-1} to more easily detect shifts in protein retention times. Other conditions appear in individual Figures.

3. Results

3.1. Reproducibility

Reproducibility of Zorbax 300SB packing lots was calculated from sample retention times on both long- and short-chain reversed-phase columns made from three different packing lots. The variability in retention times observed for a particular chromatographic system (system variability) results from contributions by both the instrument (instrument variability) and the packing. Therefore, instrument variability in the system must be accurately determined and used for reference. As a means of assessing this variability in the following experiments, retention time was used from six replicate injections made on both the HP1090 (300SB-C18 test) and HP1050 (300SB-C3 test) instruments. Mean retention times for each column lot were calculated from repeat injections on the Zorbax 300SB-C18 and 300SB-C3 columns.

To test the Zorbax 300 Å SB-C18 column, a series of five decapeptides (P1–P5) [11] were chromatographed on columns from each of three lots. These synthetic decapeptides increase in hydrophobicity in the order P1 to P5 and elute consecutively in reversed-phase chromatography; they are sensitive to changes in the column-packing surface. The chrom- atograms in Fig. 1a illustrate the consistency in peak heights and retention times for the five peptides used. This consistency applies
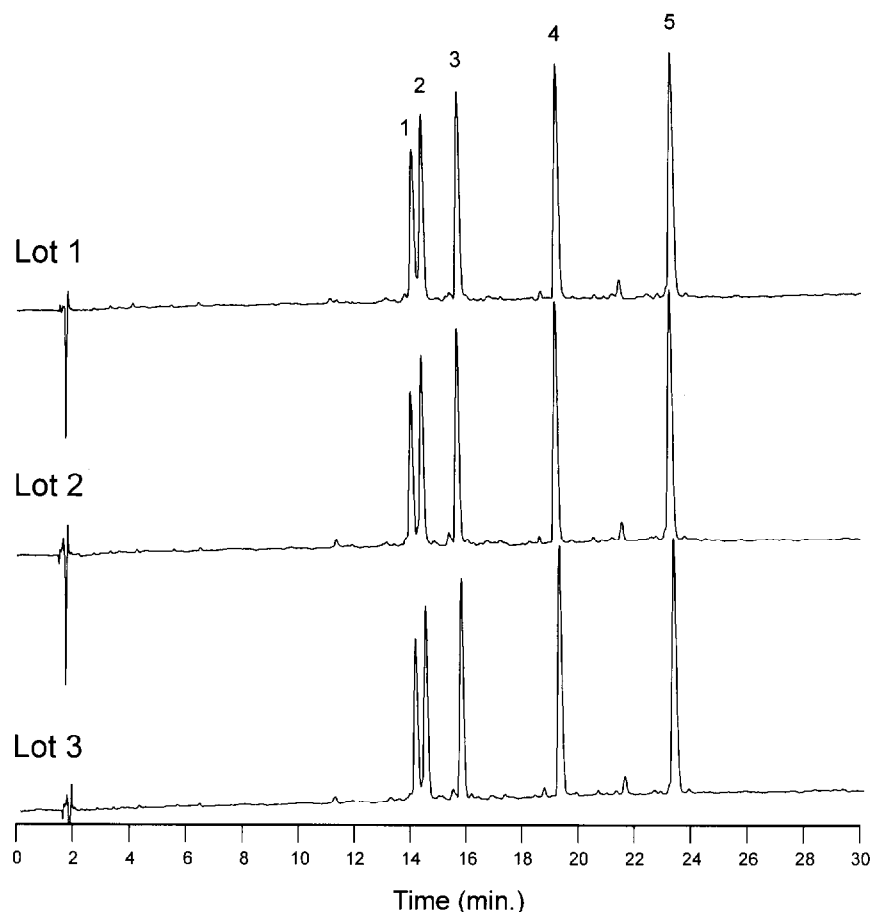
Fig. 1(a)

even to minor peaks, as evidenced by the uniform retention times and peak shapes of minor components eluting at 11.2, 18.7, 21.5, and 23.8 min.

To test the reproducibility of the Zorbax 300 Å SB-C3 column, analysis was carried out on columns from three different lots of packing. In these experiments, an eight-component mixture of peptides and proteins (see Experimental) was used to detect changes in the packing surface. The consistent retention times and peak heights (Fig. 1b) demonstrate the high reproducibility of this column packing. Again, even smaller peaks are quite reproducible, as indicated by the consistency of peaks at 12.2, 12.5 and 15 min.

Table 1a, b details results of the reproducibility test for both 300SB-C18 and 300SB-C3 packing

lots. In each case, repeat injections were made for each column (two for 300SB-C18 and three for 300SB-C3) and the mean retention time calculated. The Tables show mean peak retention times in addition to standard deviations (SD) and relative standard deviations (%RSD) between lots. Study of Table 1a confirms the excellent reproducibility in retention times for decapeptides on the 300SB-C18 column, as shown by SD values for system variability in the range 0.0082–0.0281. These values are very similar to those SD values for the instrument variability (0.0112–0.0186), indicating limited lot-to-lot variation.

Results for the eight-component sample on the 300SB-C3 column were not as straightforward as those for the experiment above. As shown in
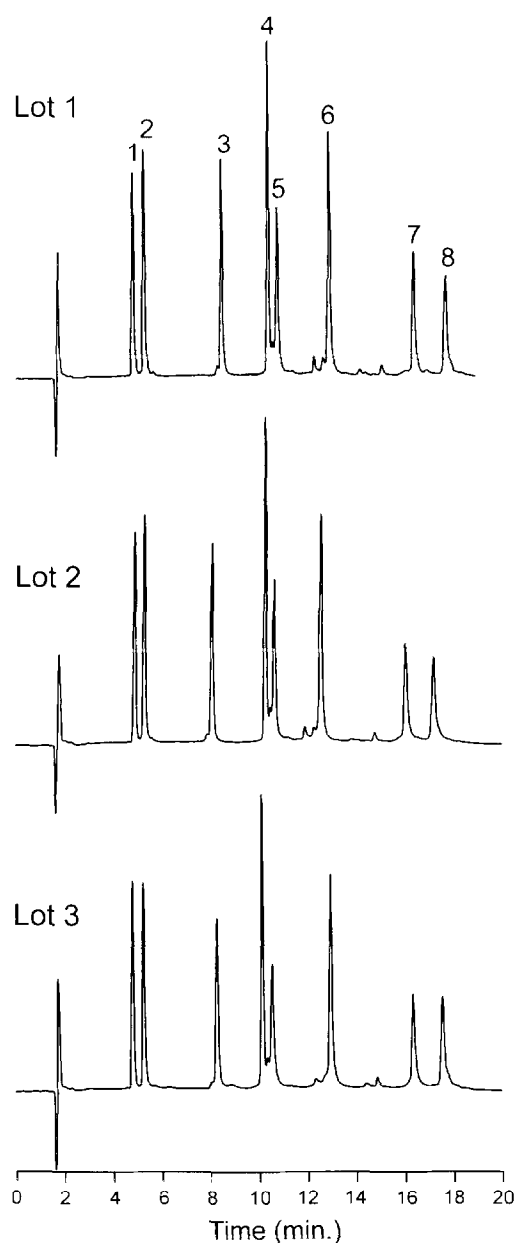
Fig. 1(b).

Fig. 1. Reproducibility of Zorbax 300SB packing lots. (a) Decapeptides of increasing hydrophobicity P1–P5 were separated on three different lots of Zorbax 300 Å SB–C18 under identical conditions. Mobile phase: H_2O–TFA (99.9:0.1, v/v) for solvent A, and ACN–TFA (99.91:0.09, v/v) for solvent B; 4–32% B in 30 min. Flow: 1.0 ml min^{-1}, UV: 210 nm, temperature: 60 °C, injections 10 μl, 6 μg of peptide. Peak 1 = P1, peak 2 = P2, peak 3 = P3, peak 4 = P4, and peak 5 = P5. (b) A series of peptides and proteins were separated on three lots of Zorbax 300 Å SB–C3 under identical conditions. Mobile phase: H_2O–ACN–TFA (95:5:0:0.10, v/v/v) for solvent A and H_2O–ACN–TFA (5:95:0.085, v/v/v) for solvent B. 15–53% B in 20 min. Flow: 1.0 ml min^{-1}, UV: 215 nm, temperature: 35 °C, injections: 10 μl, 2–6 μg of each protein. Peak 1 = leucine enkephalin, peak 2 = angiotensin II, peak 3 = RNase A, peak 4 = insulin, peak 5 = cytochrome C, peak 6 = lysozyme, peak 7 = myoglobin, and peak 8 = carbonic anhydrase.

Table 1
Reproducibility of Zorbax 300SB packing lots. (a) Reproducibility of mean retention times for five decapeptides on three 300SB–C18 packing lots. (b) Reproducibility of mean retention times for three repreat injections of eight peptides on three lots of 300SB–C3

(a)

		P1	P2	P3	P4	P5
Column-lot variation						
Inj. average	Lot 1	14.000	14.358	15.633	19.135	23.206
	Lot 2	13.960	14.334	15.610	19.116	23.192
	Lot 3	13.973	14.302	15.590	19.114	23.207
Lot average		13.977	14.331	15.611	19.121	23.202
	SD	0.0207	0.0281	0.0218	0.0119	0.0082
	%RSD	0.1479	0.1959	0.1395	0.0621	0.0355
Instrument variation						
Injection 1	Lot 3	13.971	14.303	15.59	19.116	23.211
Injection 2		13.974	14.301	15.589	19.111	23.202
Injection 3		13.964	14.301	15.554	19.104	23.193
Injection 4		13.939	14.274	15.562	19.089	23.187
Injection 5		13.962	14.292	15.579	19.103	23.196
Injection 6		13.928	14.268	15.555	19.079	23.179
Inj. average		13.956	14.290	15.577	19.100	23.195
	SD	0.0186	0.0152	0.0147	0.0139	0.0112
	%RSD	0.1329	0.1064	0.0941	0.0727	0.0484

(b)

		Leu Enk	AngioII	RNase	Ins	Cyt C	Lyso	Myo	Carb Anhy
Column-lot variation									
Inj. average	Lot 1	4.830	5.293	8.560	10.603	11.027	13.113	16.713	18.047
	Lot 2	4.860	5.270	8.200	10.290	10.630	12.563	16.080	17.277
	Lot 3	4.807	5.283	8.463	10.513	10.930	13.027	16.537	17.777
Lot average		4.832	5.282	8.408	10.469	10.862	12.901	16.443	17.700
	SD	0.0267	0.0117	0.1863	0.1613	0.206	0.2967	0.3268	0.3907
	%RSD	0.553	0.222	2.216	1.541	1.904	2.292	1.988	2.207
Instrument variation									
Injection 1		4.820	5.290	8.580	10.640	11.090	13.180	16.790	18.110
Injection 2		4.810	5.280	8.560	10.620	11.070	13.170	16.770	18.090
Injection 3		4.860	5.310	8.540	10.550	10.920	12.990	16.580	17.940
Injection 4		4.780	5.250	8.510	10.540	10.940	13.010	16.670	18.030
Injection 5		4.830	5.290	8.420	10.440	10.860	12.910	16.470	17.750
Injection 6		4.820	5.270	8.450	10.360	10.740	12.890	16.350	17.670
Inj. average		4.820	5.282	8.510	10.525	10.937	13.025	16.605	17.932
	SD	0.0261	0.0204	0.0632	0.1073	0.1313	0.1249	0.1729	0.1833
	%RSD	0.541	0.386	0.743	1.019	1.200	0.959	1.042	1.022

Leu Enk = leucine enkephalin; Angio II = angiotensin II; RNase A = ribonuclease A; Ins (BOV) = bovine insulin; Cyto C = cytochrome C; Lyso = lysozyme; Myo = myoglobin; Carb Anh = carbonic anhydrase.

Table 1b, SD values for protein retention times ranged from 0.1613 for the smallest molecule (insulin) to 0.3907 for the largest (carbonic anhydrase). This larger variability, not observed for peptides (leucine enkephalin (0.0267) and angiotensin II (0.0117), can be explained by the larger S values inherent in larger molecules (see Discussion and Conclusions). Instrument variability increased on the same order of magnitude when comparing peptides to proteins (e.g. 0.0261–0.1833) and could account for about half of the system variability.

3.2. Stability

To determine the stability of Zorbax 300 Å, sterically protected column packing, both long- and short-chain StableBond packing mater-ials (300SB-C18 and 300SB-C3), were used under extreme conditions of low pH and higher temperature. These results were compared to those of commerically available, polymerically bonded, silica-packing materials.

The stability of long-chain, alkyl bonded phases were determined under conditions of pH* 0.8 and a temperature of 90 °C. Reduction in the retention time of toluene was used as an indicator of bonded-phase degradation (Fig. 2a). Traditional packing materials lose bonded phase very rapidly, retaining only 38–70% of the original k' value for toluene after 7500 column volumes. At this point, the Zorbax 300SB-C18 column retained 100% of its original k' value for toluene, indicating no detectable loss of bonded phase. Only a 1–2% loss in k' for toluene was observed even after 26 000 column volumes.

To test the stability of short-chain alkyl-bonded phases, the columns were run at pH* 1.0 and 60 °C. The decrease in retentivity of phenylheptane (%k' remaining) was used as an indication of any loss in bonded phase. As shown in Fig. 2b, the sterically protected C3 was very resistant to hydrolysis, as shown by only a 2% loss in retention of phenylheptane after 5000 column volumes. In contrast, a traditional polymeric-C4 column lost more than 50% of its original k' value for phenylheptane after about 1400 column volumes. Steric protection of the siloxane bond joining the

silane to the silica surface results in significant protection against its loss under low-pH-high temperature conditions.

## 3.3. Changing selectivities using bonded phase and temperature

The levels of temperature stability demonstrated for the column packings in this paper are not available when using traditional bonded phases. The usable range of separation conditions is therefore reduced, limiting the ability of the chromatographer to achieve a desired separation. Stable short-chain bonded phases also mean that selectivity can be achieved through change to additional bonded phases wihtout the traditional loss of method reproducibililty. Experiments were carried out to demonstrate the use of the bonded phase and temperature change to change selectivity of proteins and peptides.

To characterize the effect of temperature change on resolution, separations of a series of peptides and proteins were performed on a Zorbax 300SB-C3 column at several temperatures from ambient to 60 °C (Fig. 3). The chromatograms show that overall separation of these proteins and peptides is generally similar for various temperatures. As expected, the retention times of nearly all the proteins and peptides decrease with increasing temperature. Closer examination of the data reveals several significant changes. First, peaks 4 and 5 (insulin and cytochrome C respectively) cross over as temperature is increased. This occurs as a result of decrease in retention of cytochrome C while retention of insulin increases slightly. (Carbonic anhydrase is the only other peak that increases in retention). Note specifically that peaks 4 and 5 are completely overlapped at 45 °C.

Resolution of components in a chromatographic sample is controlled by the relative difference in retention times (t_R) and by efficiency, or peak widths, of the components at half the peak height ($PW_{1/2}$). The ratio of k' values, or selectivity (α), is calculated as k'_n/k'_{n-1}, where $k' = (t_R/t_0 - 1)$ for peak n, t_R is the component retention time, and t_0 is the column void volume. Table 2 is
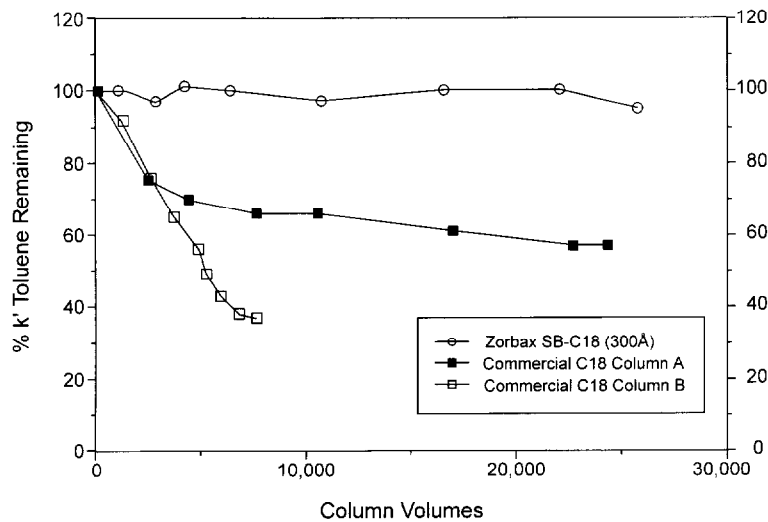
(a)
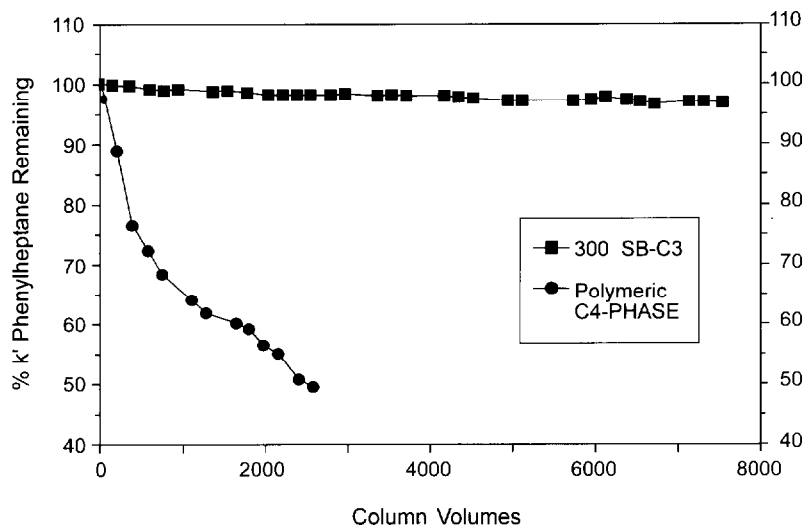
(b)

Fig. 2. Stability comparison of sterically protected and traditional bonded phases at low pH and high temperature. (a) Zorbax 300SB–C18 and two commercially available C18 columns were tested for stability by continuously purging with MeOH–H$_2$O–TFA (50:49:1.0, v/v/v), (pH* 0.8, 90 °C). Toluene, detected at 254 nm, was used as a test probe for loss of bonded phase. (b) Zorbax 300SB–C3 and a commercially available C4 column were tested for stability by repeated ACN–TFA gradients at pH 1 and 60 °C as described in the Experimental. Phenylheptane was injected isocratically at 40 °C, ACN–H$_2$O–TFA (70:30:0.1, v/v/v), and detected at 254 nm to serve as a marker of bonded-phase loss.

a compilation of peak width and selectivity values for sample components in the temperature experiments of Fig. 3. As expected, peak widths decrease with temperature, and increase with sample molecular weight. However, since selectivity dominates resolution, increase in resolution by reducing in peak width may be offset by an unfavorable change in selectivity. This is the case for cytochrome C and insulin; for all other components in this sample, peak widths and selectivity work synergistically to improve resolution at increased temperature.
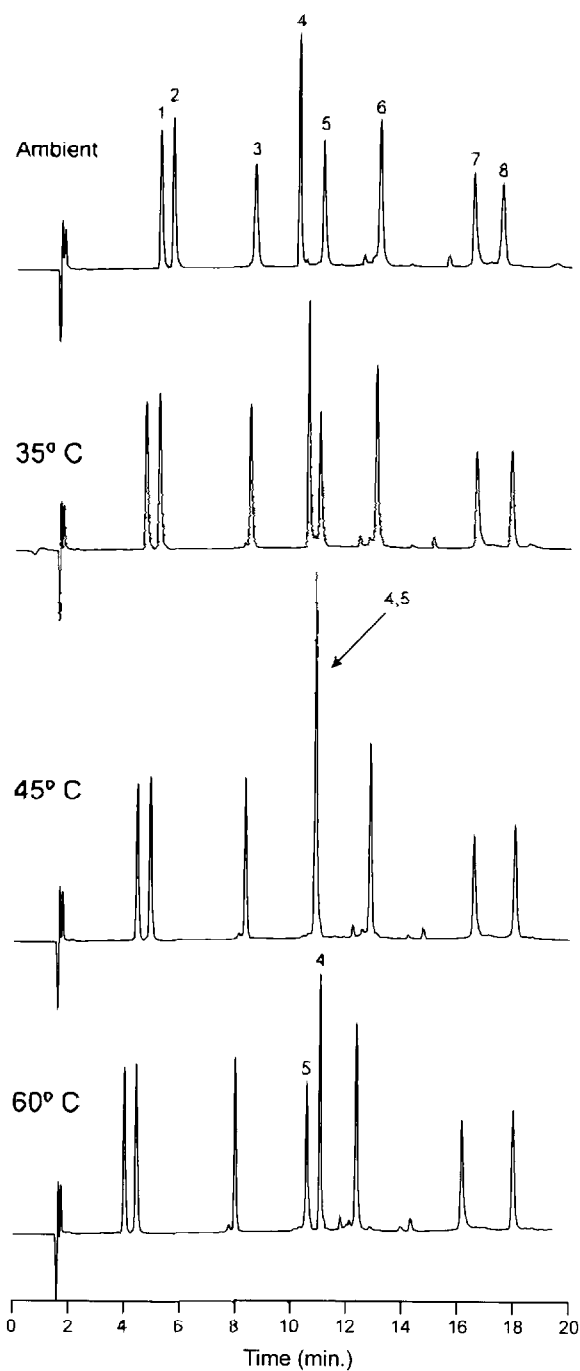
Fig. 3. Effect of temperature on peak-widths and seletivity of peptides and proteins. A series of polypeptides were chromatographed on a Zorbax 300 Å SB–C3 column at increased operational temperatures. Mobile phase: H_2O–ACN–TFA (95:5:0.10, v/v/v) for solvent A and H_2O–ACN–TFA (5:95:0.085, v/v/v) for solvent B, 15–53% B in 20 min. Flow: 1.0 ml min^{-1}, UV: 215 nm, temperature: as stated in Figure, injections: 10 μl, 2–6 μg of each protein. Peak 1 = leucine enkephalin, peak 2 = angiotensin II, peak 3 = RNase A, peak 4 = insulin, peak 5 = cytochrome C, peak 6 = lysozyme, peak 7 = myoglobin, and peak 8 = carbonic anhydrase.

Table 2

Effect of temperature on peak-widths and retention of polypeptides. A series of peptides and proteins were chromatographed on Zorbax 300 Å SB-C3 at increasing temperatures. Peak widths at half-height (PW1/2) and alpha values of successive peaks are shown

Polypeptide	MW	PW1/2				α			
		Ambient	35 °C	45 °C	60 °C	Ambient	35 °C	45 °C	60 °C
Leu Enk	556	0.097	0.090	0.086	0.080	#N/A	#N/A	#N/A	#N/A
Angio II	1046	0.099	0.094	0.092	0.089	1.12	1.15	1.17	1.18
RNaseA	12 600	0.130	0.091	0.081	0.074	1.73	1.91	2.04	2.26
Ins(BOV)	5700	0.082	0.079	0.086	0.074	1.23	1.31	1.38	1.48
CytoC	12 400	0.098	0.089	0.086	0.076	1.10	1.05	1.00	0.95
Lyso	14 400	0.120	0.093	0.087	0.081	1.21	1.22	1.21	1.20
Myo	16 900	0.121	0.116	0.108	0.098	1.29	1.32	1.33	1.35
CarbAnh	29 000	0.149	0.126	0.115	0.101	1.07	1.08	1.10	1.13

Leu Enk = leucine enkephalin; Angio II = angiotensin II; RNaseA = ribonuclease A; Ins(BOV) = bovine insulin CytoC = cytochrome C; Lyso = lysozyme; Myo = myoglobin; CarbAnh = carbonic anhydrase.

Experiments examining the effect of bonded-phase change on separations of peptide and proteins were carried out using the Zorbax 300SB-CN, -C3, -C8, and -C18 columns. Effect of increasing temperature was again studied, by running the separations at both 35 and 60 °C. To provide accuracy and readily observable results, three sample groups were chosen that had closely eluting peaks. The sample groups consisted of: (1) leucine enkephalin, methionine enkephalin, neurotensin, and angiotensin II; (2) insulin, insulin B, and cytochrome C; and (3) myoglobin, calmodulin, and carbonic anhydrase. Chromatograms for all three samples on four bonded phases are shown for 35 °C in Fig. 4a and for 60 °C in Fig. 4b. Multiple chromatograms have been combined to simplify comparisons and save space.

First, a comparison of results for sample group 1 shows that baseline resolution of peaks 2 and 3 only occurs by using the Zorbax 300SB-C3 column. Separation of peaks 2 and 3 on the other bonded phases was very similar. For this sample, temperature change resulted in no improvement of resolution on any bonded phase (compare peaks 1–4; Fig. 4a, b).

Sample group 2 (insulin, insulin B, and myoglobin) provided the most challenging separation and showed significant differences in chromatography with change in bonded phase and temperature change. The Zorbax 300SB-CN and -C3 columns demonstrate the best separation for this sample at 35 °C (peaks 5–7, Fig. 4a). Chromatography on the 300SB-C8 and -C18 columns resulted in overlap of two of the three peaks. Separations at 60 °C (peaks 5–7, Fig. 4b) showed a large selectivity change for peak 5 on all four columns, resulting in its improved resolution from other components. Also at 60 °C, peaks 6 and 7 were overlapped on the 300SB-C3 and -C8 columns, while all three components were well resolved on the 300SB-C18 column. Chromatography on the 300SB-C18 column at elevated temperature resulted in high resolution of all three peaks; however, peak 7 (cytochrome C) had poor peak shape (see Discussion and Conclusions). The proper elution time and peak shape of cytochrome C were verified by single-component injection on the other bonded phases (data not shown).

Finally, sample group 3 (myoglobin, calmodulin, and carbonic anhydrase) demonstrates the combined use of bonded phase and temperature change to effectively change selectivity of large proteins (peaks 8–10, Fig. 4a b). At 35 °C, near-baseline resolution of all three components is shown by the 300SB-C18 column. The components are separated to a lesser extent on the 300SB-CN and -C8 columns. Elevation of the
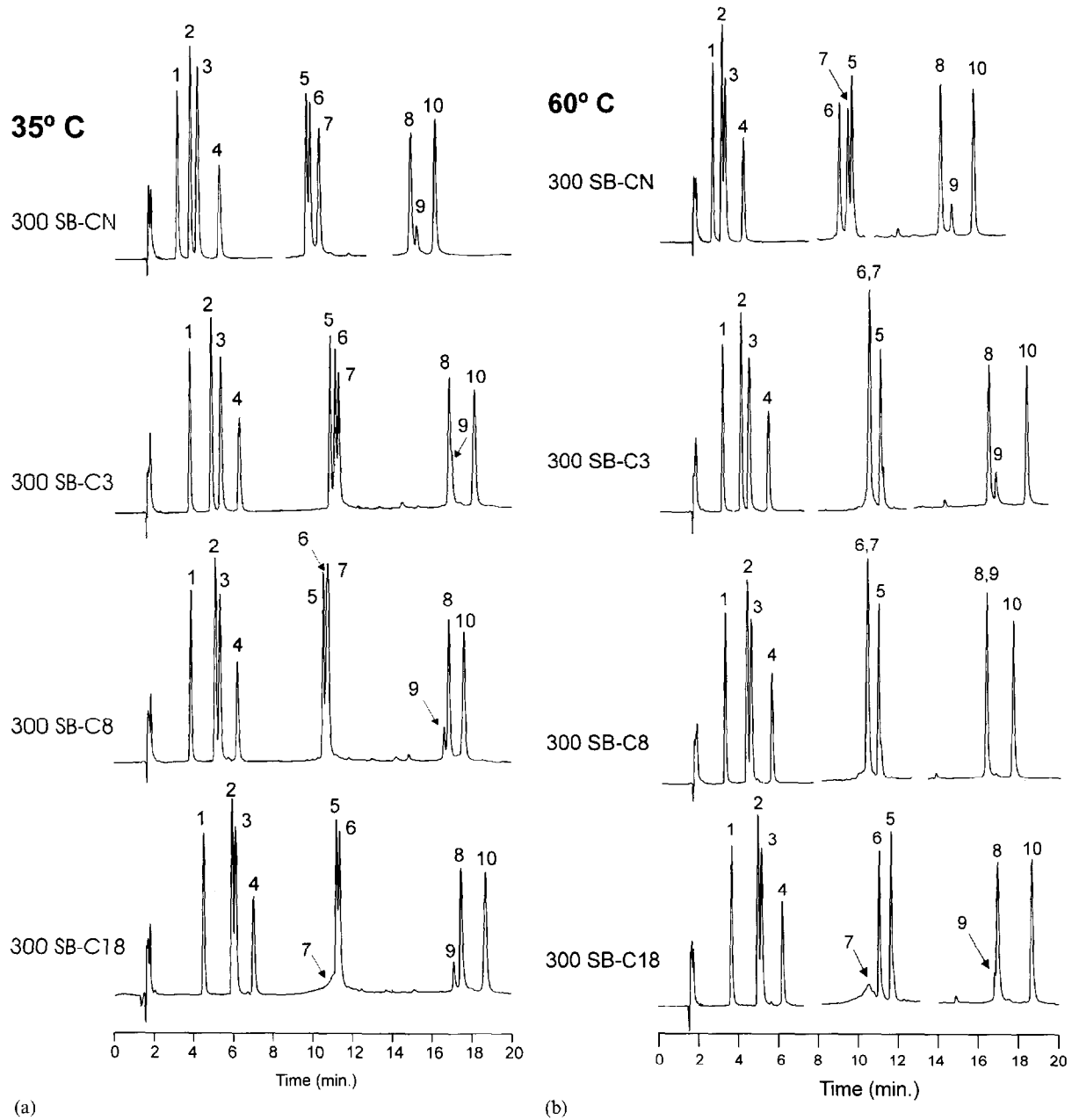
Fig. 4. Effect of bonded phase and temperature on resolution of three groups of closely eluting peptides and proteins: (a) 35 °C; (b) 60 °C. Each group was separated on four Zorbax 300SB phases (Zorbax 300SB–CN, -C3, -C8, and -C18). Mobile phase: H_2O–ACN–TFA (95:5:0.10, v/v/v) for solvent A and H_2O–ACN–TFA (5:95:0.085, v/v/v) for solvent B, 15–53% B in 20 min. Flow: 1 ml min^{-1}, UV: 215 nm, temperature as stated, injections: 10 µl, 2–6 µg of each protein. Group 1: peak 1 = methionine enkephalin, peak 2 = leucine enkephalin, peak 3 = angiotensin II, peak 4 = neurotensin. Group 2: peak 5 = insulin, peak 6 = insulin B chain, peak 7 = cytochrome C. Group 3: peak 8 = myoglobin, peak 9 = calmodulin, peak 10 = carbonic anhydrase.

operating temperature to 60 °C results in improvement of the separation to near-baseline for

both the 300SB-CN and -C3 columns. It should be pointed out that optimal conditions for separa-

tion to near-baseline for both the 300SB-CN and
-C3 columns. It should be pointed out that
optimal conditions for separation of a specific
component from others are often not those for
optimal separation of all the sample components
(e.g. separation of insulin or carbonic anhydrase
from their group components).

4. Discussion and conclusions

Examples of the need for both stability and
reproducibility in the reversed-phase HPLC of
peptides and proteins are extensive. They include
separations such as identification of wheat vari-
eties by patterns of protein peaks, analysis and
quantitation of pharmaceutical products, and ba-
sic research studies of peptides and proteins. In
each of these cases, increased reproducibility and
stability result in decreased cost, reduced separa-
tion time, and improved reliability of results.
Data discussed in this paper show extreme repro-
ducibility and stability for Zorbax 300 Å column
packings. As a result of this stability, achievable
even for short-chain bonded phases, two addi-
tional reversed-phase chromatographic tools be-
come available. These are the ability to achieve
resolution by changing to reliable, short-chain
bonded phases, and the ability to achieve resolu-
tion through higher operating temperatures.

In reproducibility tests, it was found that reten-
tion times for peptides were much more repro-
ducible than those for proteins. Table 1 shows
that the standard deviation (SD) of successive
runs was about 0.0150. However, for larger
polypeptides and proteins, variabilities were on
the order of ten-fold greater. The increase in
variability for these larger molecules is expected
because of the significant increase in S values. S
values are derived from the slope of plots of log
retention time versus organic solvent concentra-
tion. Retention times of larger molecules are
much more sensitive to change in solvent strength
of the mobile phase. Instrument-related variation
in retention times increased consistently with sam-
ple molecular weight. This also, can be attributed
to increased S values since consistent elution of
larger molecules requires additional accuracy of

mobile-phase solvent strength and delivery.
Proper instrument function in the protein experi-
ment is verified by the similar SD values for
repeat injections of peptides (compare 0.0261 and
0.0204 to 0.0186). Protein values were on the
order of 10 times greater than those for peptides.

In addition to expected sources of variability,
there was an additional source that correlated
with system performance. The chromatograph
used to collect data on the 300SB-C3 column
(HP-1050) had a tendency to collect bubbles in
the proportioning value, mixer, or piston area.
Although variation in pump pressure did not
result in pump shutdown, it led to significant
changes in the retention times of larger proteins.
To avoid this problem, it was necessary to care-
fully sparge the system with helium several times a
day, in addition to normal sparging and use of
covered mobile-phase containers. This aggressive
sparging can pose a problem with loss of TFA
due to its volatility and additional shifts in peak
retention.

As a result of the increased stability and repro-
ducibility available from Zorbax 300 Å Stable-
Bond columns, changes in bonded-phase
chemistry and changes in temperature can be
employed as tools to effect difficult separations.
Not only does this type of change make sense
from the standpoint of altering selectivity of the
separation, but it is also one of the easiest changes
to make in a chromatography system—requiring
no change in mobile phase or gradient. It is clear
from the data that changing the bonded phase
does not always improve the separation. This is
especially true for changes from C18 to C8, where
the bonded phase characteristics are very similar.
However, bonded-phase changes from a very po-
lar short-chain phase (SB-CN) to a non-polar
longer-chain phase (SB-C8 or SB-C18), or vice
versa, results in much more significant changes in
selectivity. In these limited numbers of experi-
ments, there were several cases where this type of
change caused significant improvement in selectiv-
ity, that would not easily, or at all, be achievable
using changes in mobile phase. Notice that the
300SB-C3 column (tri-isopropyl functional group)
demonstrates certain selectivity advantages over
other bonded phases.

As mentioned in the Results, the 300SB-C18 column was the only one showing the poor chromatography of cytochrome C (peak 7, Fig. 4a b). Although cytochrome C has a high pl (overall positive charge at pH < 9.6), it is also demonstrates strong hydrophobic character when solubilized in organic solvents. In addition, the 300SB-C18 column has a higher phase ratio than the other, less hydrophobic, bonded phases. It is logical to conclude that this peak broadening is due to strong hydrophobic interaction of cytochrome C with the column packing. Other results (not shown) indicate that this tendency increases with molecular weight of the protein.

Because of the increased stability of the Stable-Bond packing materials, increased temperature is now also a tool for effecting separations. As with bonded-phase change chromatography was better at lower temperatures, in many cases; however, there was significant improvement in several cases in separation of closely eluting compounds or overlapping compounds, achieved by a simple elevation of temperature. Finally, as packing materials improve and new technologies are developed, chromatographers will enjoy additional tools for achieving separations, as a benefit of these advances.

Acknowledgments

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