Journal of Chromatography, 499 (1990) 125–139
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 787

FACTORS INFLUENCING THE PERFORMANCE OF PEPTIDE MAPPING BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

MICHAEL W. DONG*
The Perkin-Elmer Corporation, 761 Main Avenue, Norwalk, CT 06859-0250 (U.S.A.)
and
AN D. TRAN
Ortho Pharmaceutical Corporation, Raritan, NJ 08869-0602 (U.S.A.)

SUMMARY

Factors controlling the performance of peptide mapping on reversed-phase columns were systematically evaluated. Performance criteria included resolution (peak capacity and selectivity), system reproducibility, sensitivity and analysis speed. Column configuration, characteristics of packing materials, mobile phase composition, operating variables and instrumental designs were found to influence the performance of peptide mapping. Considerations for peptide identification techniques are discussed.

INTRODUCTION

Peptide mapping is a powerful method for the structural identification of proteins[1,2]. In peptide mapping, a sample protein is selectively cleaved by enzymes (*e.g.*, trypsin, chymotrypsin, endoproteinase Lys-C, V8 proteinase from *Staphylococcus aureus*, pepsin, subtilsin, clostripain, etc.) or by chemical digestions (*e.g.*, cyanogen bromide). Trypsin, the most common agent, cleaves the carboxyl side of the peptide bonds of lysine, arginine and aminoethyl cysteine and forms many fragments averaging 7–12 amino acid residues[3]. In contrast, cyanogen bromide cuts the protein at the methionine site and yields relatively large fragments.

The digest is then analyzed by liquid chromatography to yield a peptide map. Reversed-phase high-performance liquid chromatography (RP-HPLC) is the primary mode used because of its high resolution and selectivity[4]. The resultant peptide map is a unique "fingerprint" profile of the protein and may be compared to a reference chromatogram to establish equivalency.

Fig. 1 shows two comparative tryptic maps of recombinant tissue-type plasminogen activator (rt-PA), a glycosylated protein of approximately 64 000 daltons which was recently approved by the Food and Drug Administration (FDA) for treating heart attack patients[5–7]. The bottom map of a mutant rt-PA is virtually identical to the reference map except the retention time of one peptide is shifted. This is due to the substitution of glutamic acid for arginine in position 275. Such a variance in peak retention is used to identify changes in the amino acid sequence of the protein.

0021-9673/90/$03.50

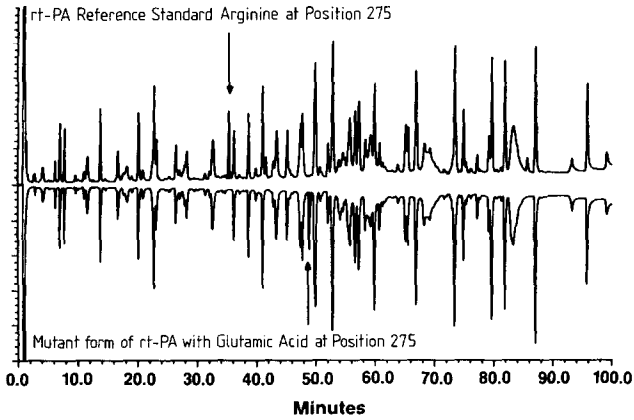

Fig. 1. Tryptic map chromatograms of the rt-PA reference standard and a mutant form of rt-PA with a glutamic acid residue in place of the normal arginine residue at position 275. Arrows illustrate the differences in the two chromatograms caused by the substitution. Courtesy of Garnick *et al.*, from ref. 6.

Peptide mapping is the premier method for the structural elucidation of newly discovered proteins. Since 80–90% of the eukaryotic proteins cannot be sequenced directly due to blocked amino-termini[8], peptide mapping is used to isolate fragments for sequence analysis. The technique is also used to locate glycosylation sites[9,10] and disulfide linkages[11]. Today, peptide mapping is increasingly used in biotechnology for the quality control of recombinant proteins. Mapping provides vital information on lot-to-lot product consistency, expression errors, and mutation or deamidation sites[5,6].

Peptide mapping is one of the most demanding applications of HPLC. A typical map contains 20–150 peaks, all of which should be totally resolved (or near totally resolved). A high level of column resolution and system precision is required to accurately reproduce the maps, often in sub-nanomole quantities. This places stringent demands on both the compositional and flow repeatability of the solvent delivery system and also the sensitivity and stability of the detection system.

This paper describes a systematic study of the factors controlling the performance of peptide mapping by RP-HPLC. Performance criteria include resolution, system reproducibility, sensitivity and analysis speed. Factors (column, operating conditions and instrumentation) leading to optimized peptide mapping as well as considerations for sequence analysis and peptide identification are discussed.

EXPERIMENTAL

Apparatus

The LC system used in this study consisted of a Model 250 binary LC pump, a Rheodyne Model 7125 injector, an LC-135 dual-channel diode array detector, and a GP-100 printer/plotter. In addition, an LC-101 oven for column temperature control and an ISS-100 automatic sampler were used for evaluating system reproducibility. Solvent degassing was accomplished by helium sparging and pressurization in polyethylene solvent bottlers (2-l and 4-l capacity). All equipment was from Perkin-Elmer (Norwalk, CT, U.S.A.).

Columns

Vydac columns packed with polymeric C_{18} bonded phase were used. These columns, available from Perkin-Elmer or from the Separations Group (Hesperia, CA, U.S.A.) had the following dimensions: 250 mm × 4.6 mm I.D.; 150 mm × 4.6 mm I.D.; 50 mm × 4.6 mm I.D. and 250 mm × 2.1 mm I.D. The packings had nominal particle diameter and pore size of 5 μm and 300 Å, respectively. An experimental column (83 mm × 4.6 mm I.D.) packed with 3-μm C_8 materials was also evaluated.

A scavenger column (33 mm × 4.6 mm I.D.) packed with 10-μm C_{18} particles placed between the pump and the injector protects the analytical column from mobile phase contaminants[12]. This precolumn eliminates the need for solvent filtration through membrane filters, which can introduce gradient artifacts[13], and also helps to prolong column lifetime. A low-capacity guard column (30 mm × 2.1 mm I.D.) dry-packed with 40-μm C_{18} pellicular materials was also found to be satisfactory in offering protection against sample contaminants without adversely affecting performance of the analytical columns[12].

Chemicals and reagents

All sample proteins, L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, and trifluoroacetic acid (TFA) were obtained from Sigma (St. Louis, MO, U.S.A.). Other chemicals and reagents were obtained from Aldrich (Milwaukee, WI, U.S.A.) and Fisher Scientific (Fairlawn, NJ, U.S.A.). HPLC-grade acetonitrile was obtained from EM Science (Gibbstown, NJ, U.S.A.). Water was filtered and purified by passage though mixed-bed, ion-exchange, and activated charcoal cartridges.

Mobile phase preparation

Three mobile phase systems were used:

Mobile phase system	*Strong solvent (A)*	*Weak solvent (B)*
(I) 0.1% TFA	0.1% TFA acetonitrile	0.1% TFA in water
(II) Balanced absorbance	0.054% TFA in acetonitrile–water (80:20)	0.06% TFA in water
(III) Phosphate	Acetonitrile	50 m*M* NaH_2PO_4 (pH 2.6)

Most proteins/peptides yield better peak shapes under acidic pH[14]. The use of 0.1% TFA with acetonitrile has been adopted by most analysts as the "standard" mobile phase for RP-HPLC of proteins. However, use of mobile phase system I (0.1% TFA) is limited to low sensitivity applications because baseline shifts due to unbalanced absorbance preclude its use in mapping below 250 pmol levels.

Procedure for preparing tryptic digests

Reduction and S-carboxymethylation of proteins were carried out according to the procedure of Crestfield *et al.*[15] with the exception that mercaptoethanol was replaced by dithiothreitol (DTT)[16]. The procedure works well for milligram quantities of protein. However, lower levels are problematic because of the loss of proteins during the dialysis and lyophilization steps. An alternate tryptic digestion procedure suitable

for subnanomole levels of proteins in which these low yield steps were replaced with a dilution step with water, was used for the human transferrin sample[17].

RESULTS AND DISCUSSION

Table I lists the performance criteria and the key factors controlling the performance of peptide mapping by liquid chromatography. These factors fall into three distinct categories: column parameters, operating conditions and instrumentation. Each performance criterion and its influencing factors are discussed in detail below.

Resolution

The primary goal in mapping is the resolution of all peptide fragments. Since their size and hydrophobicity vary, gradient elution techniques are used. Resolution is a function of column efficiency (N), retention (k'), and selectivity (α). In complex maps in which all peaks need to be resolved, the overall peak capacity is the most important criterion[18]. In contrast, for resolving co-eluting peaks, increasing the chromatographic selectivity by changing the gradient time (t_G), the mobile phase or the bonded phase is often sufficient. Above all, the peptide columns used must yield symmetrical peak shapes with quantitative mass recovery for all fragments.

Peak capacity. Peak capacity is an empirical parameter defined as the maximum number of peaks that can theoretically be resolved in the chromatogram. Peak capacity is calculated by dividing the net retention time of the last peak in the chromatogram by the average peak width[18]. It is primarily a function of N, gradient time (t_G), and flow-rate (F).

Fig. 2 shows how peak capacity varies with t_G and column length (L). Three Vydac C_{18} columns of 5, 15 and 25 cm length were used in the experiments. Peak

TABLE I

FACTORS INFLUENCING THE PERFORMANCE OF PEPTIDE MAPPING BY LC

Criteria	*Key factors*
Resolution	Column efficiency
Column peak capacity	Flow-rate and gradient time
Selectivity	Mobile phase, bonded phase, gradient time
Peak shape and mass recovery	Silica type, pore size
System reproducibility	Pump flow and gradient precision
	Temperature fluctuations
	LC system reliability
	Column lot-to-lot reproducibility and lifetime
Detection sensitivity	Detector noise and drift
	Mobile phase purity and absorbance
	Monitoring wavelength
	Column diameter
	Flow-rate
Analysis speed	Column length, particle size
	Flow-rate and gradient time

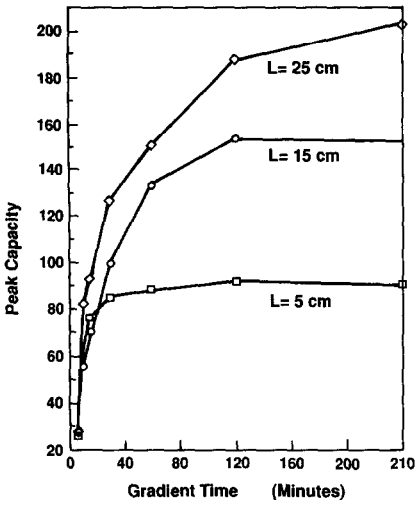

Fig. 2. Graph showing the variation of peak capacity *vs.* gradient time and column length (*L*.). Columns were packed with 5-μm Vydac C_{18} materials. Mobile phase: A = 0.1% TFA in acetonitrile, B = 0.1% TFA in water; Gradient conditions: 2% A to 32% A, 1 ml/min and 40°C; sample = lysozyme digest. Peak capacities were calculated from peaks 4, 8, 12 and 15 of the digest (see Fig. 6a).

capacity was found to increase with column length and gradient time. The lower curve (5-cm column) shows that peak capacity increases rapidly with t_G up to about 30 min and flattens out beyond 40 min to a value of 90. Fig. 2 also demonstrates that to achieve high peak capacities (>150), longer columns (15 or 25 cm) should be used with gradient times of 80–120 min. For gradient times beyond 2 h, peak capacities appear to reach a constant level for the 15-cm column and a point of diminishing return for the 25-cm column (at 1 ml/min).

Fig. 3 shows a high-resolution tryptic map of human transferrin (unmodified molecular weight 75 000 daltons) using a 25-cm column and a t_G of 95 min at 1 ml/min. The complexity of the chromatogram is typical for tryptic maps of larger proteins. As Fig. 2 indicates, these operating conditions are near optimum for the 5-μm Vydac columns used.

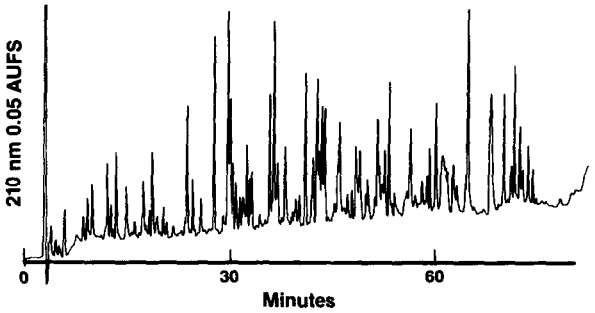

Fig. 3. High-resolution tryptic map of human transferrrin (140 pmol). LC conditions: column: 5-μm Vydac C_{18} (250 × 4.6 mm I.D.). Mobile phase: A = 0.056% TFA in acetonitrile–water (80:20); B = 0.06% TFA in water; gradient conditions 2–37% A in 63 min, 37–75% A in 32 min, 75–98% A in 10 min, 1 ml/min, ambient temperature, pressure drop 1200 p.s.i. From ref. 35.

A systematic treatment relating peptide resolution to gradient parameters is found elsewhere[19]. Gradient parameters have a profound effect on peptide resolution because they control average retention ($\bar{k}$) as shown in eqn. 1[19].

$$\bar{k} = \frac{t_G\ (F)}{1.15\ (\Delta\varphi)\ V_m\ S} \tag{1}$$

Where $\bar{k}$ = Average solute capacity factor under gradient
φ = Fraction of organic solvent in the mobile phase
$\Delta\varphi$ = Change of φ during gradient
V_m = Column void volume
S = Solute parameter (slope of log k' *vs.* φ)

$\bar{k}$ describes the average solute retention under gradient conditions and is analogous to k' for isocratic separations. Eqn. 1 indicates that an increase in t_G (holding F constant) leads to higher values of $\bar{k}$. Higher $\bar{k}$ is equated to higher resolution (or peak capacity) since greater volumes of a lower strength mobile are passed through the column. This phenomenon was confirmed by data presented in Fig. 2.

Fig. 4 demonstrates the relationship between peak capacity and $\bar{k}$ at different flow-rates for the 15-cm Vydac column. Peak capacity was found to increase with $\bar{k}$ (1–40) over a flow-rate range of 0.5 to 3 ml/min. Fig. 4 also shows that for constant retention (*e.g.*, $\bar{k}$ = 10), peak capacity decreases as flow-rate increases. This is caused by a loss of column efficiency at high flow-rates due to the low diffusivities of peptides (see Fig. 9). The highest value of peak capacity in the graph (peak capacity = 168) was

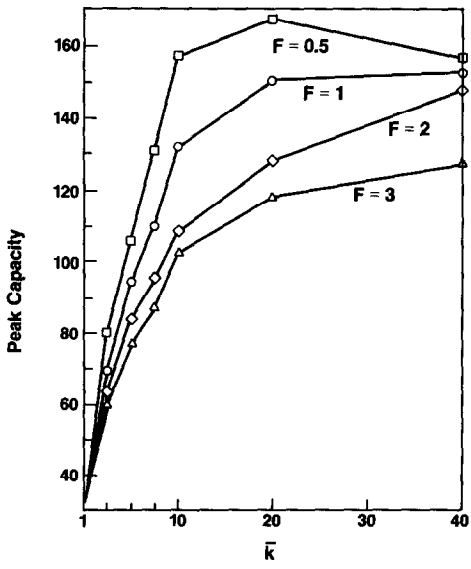

Fig. 4. Graph showing the variation of peak capacity *vs.* $\bar{k}$ using different flow-rates. Column: 5-μm Vydac C_{18} (150 mm × 4.6 mm I.D.). Mobile phase and sample were identical to that used in Fig. 2.

achieved at $F = 0.5$ ml/min and $\bar{k} = 20$ ($t_G = 208$ min). Thus, for high resolution mapping, these data suggest the use of 15 to 25 cm column lengths, operating at flow-rates of 0.5 to 1 ml/min and gradient times between 60 and 200 min or $\bar{k}$ between 10 and 20.

Selectivity. Chromatographic selectivity (α) is primarily a function of the mobile phase composition (*e.g.*, ion-pairing reagent, pH, organic solvent)[14] and the nature of the bonded phase (*e.g.*, C_4, C_8, C_{18}, phenyl)[20,21].

Fig. 5 shows two comparative tryptic maps of β-lactoglobulin A (molecular weight 34000 daltons) using different mobile phases. The figure illustrates how co-eluting fragments can be resolved completely by just changing the mobile phase. Further more, compared to 0.1% TFA (system I), phosphate buffer (system III) yields sharper peaks and less gradient baseline shift due to its higher transparency at 220 nm[22,23].

The use of bonded phases with shorter alkyl chains (*e.g.*, C_4–C_8) often yields sharper peaks and different selectivity than C_{18} phases for hydrophobic peptides[21,22]. However, the retention of small or hydrophilic peptides found in many tryptic digests is also lower in these phases.

Peak symmetry and peptide mass recovery. Peptide separations are sensitive to silica types[23–25]. The purity, acidity and residual silanophilic activity of the silica material appear to be the critical factors. Residual silanols interact with basic amino acid groups and cause peak tailing or even adsorption. The suitability of bonded phases for peptide mapping can be assessed by basic test probes such as N,N-diethyl-aniline[26] or standard peptides[27]. Alternatively, the peptide map on a new column can be compared to that of a known reference column (*e.g.*, Vydac C_{18}) for the total number of peaks and peak symmetries. Pore size of packing materials should be 125–300 Å[6] for peptides to prevent band broadening due to "restricted diffusion"[19,23]. Wide-pore packings (*i.e.*, 300 Å) are preferred for partial digests or if substantial levels of undigested proteins are present in the sample.

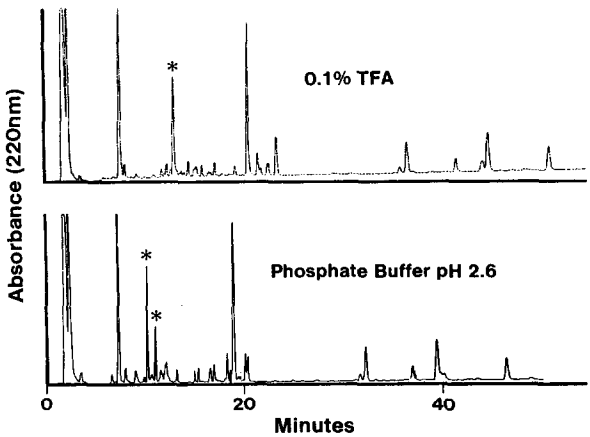

Fig. 5. Comparative tryptic maps of β-lactoglobulin A using mobile phase system I (upper) and III (lower). Gradient conditions: 0–15% A in 15 min, 15–30% A in 40 min, 1 ml/min at 40°C. The peak marked with an asterisk in the upper chromatogram was resolved in the bottom chromatogram by changing chromatographic selectivity.

Optimizing resolution in peptide mapping. The utility of predictive modeling in gradient optimization of peptide mapping has been demonstrated[28,29]. Primary structures of individual fragments of known proteins, best separation mode and retention order can also be predicted[30,31]. For very complex maps, the use of sequential digestions[32] and combined ion-exchange/reversed-phase chromatography[33,34] might be required to resolve all peaks.

System reproducibility

In peptide mapping, shifts in retention time might be interpreted as a variance of primary protein structure[6]. Thus, retention reproducibility of the LC system is a prime prerequisite. Shallow gradients (0–30% acetonitrile in water in 60–120 min) under low flow (0.2–1 ml/min), conditions that are optimal for mapping, are difficult to reproduce for many LC pumps[6,26,35,36]. Inaccurate mixing at low percentages of organic solvent (0–10%) is exacerbated by peptides which have steep elution isotherms or large S values[23].

Retention time reproducibility can be evaluated by calculating the standard deviation of retention time of selected fragments from 6–10 repetitive runs or by visual inspection of the plotted chromatograms in an overlay (Fig. 6A) or splitscreen format (Fig. 6B). In our study, relative standard deviations (R.S.D.) of 0.1 to 0.25% in retention times were demonstrated under various operating conditions (F = 0.3–2.5 ml/min and t_G = 10–95 min). These experiments were conducted with thermostatted columns at 40°C to eliminate column temperature fluctuations and with pressurized solvent reservoirs (since even gentle continuous helium sparging tends to reduce TFA levels in the mobile phase).

While short-term reproducibility is attained with precise LC pumps[35,36], long-term reproducibility is dependent on factors not easily controlled by the analyst (*e.g.*, long-term LC system reliability, column stability and lifetime, and batch-to-batch reproducibility of the packing materials).

Column batch-to-batch reproducibiliy is dependent on the exactness of the silica manufacturing process and the quality control procedures of the vendor. Column stability and lifetime can be extended by judicious column maintenance and washing procedures[23], sample cleanup techniques and the use of scavenger and guard columns[12].

Limited data suggested a column life expectancy of 200–300 injections or 10–30 l of mobile phase[18,37]. The shortened column life (compared to several thousand injections for general RP-HPLC[38] might be caused by the prolonged use of an acidic mobile phase (0.1% TFA, pH 2) which can cleave the bonded groups or the end caps, exposing the silanols that cause peak tailing[23]. This problem might be addressed via the use of polymerized C_{18} phases (*e.g.*, Vydac), novel bonding chemistries[39] and other proprietary treatment techniques (*e.g.*, Phase Separations pH-stable phases).

Sensitivity

Sensitivity is important in biochemical research because of the limited amount of sample protein available for characterization. Since digestion procedures for sub-nanomoles of proteins have previously been demonstrated[17] and sequencing can be performed routinely at the low picomole levels[40], enhancing the detection sensitivity in peptide mapping is therefore the next logical step.

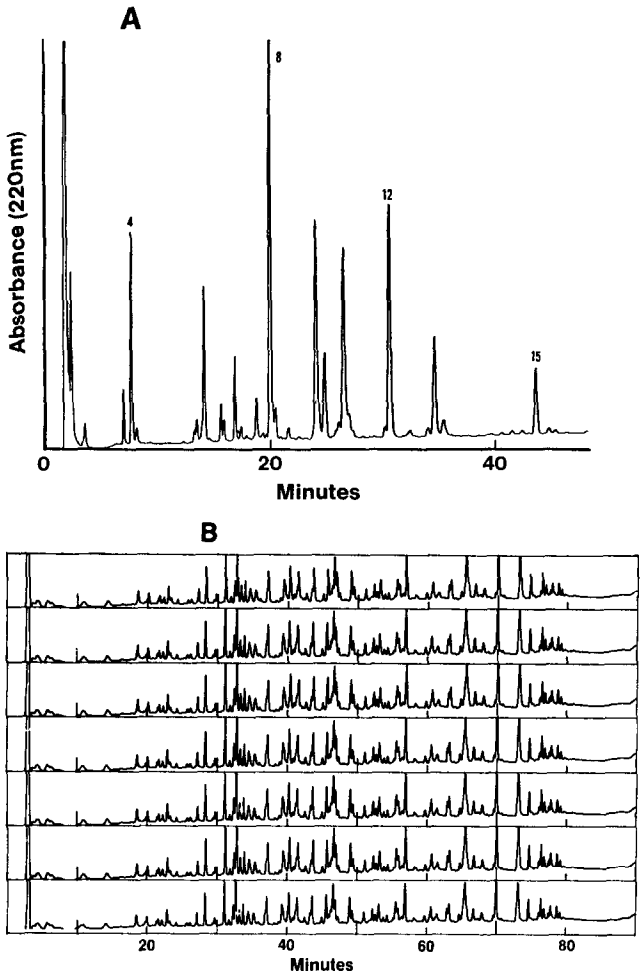

Fig. 6. System reproducibility. (A) an overlay of six tryptic maps of lysozyme. Column and mobile phase conditions identical to those used in Fig. 5 (upper). Retention time R.S.D. < 0.25%; peak area R.S.D. < 0.4%. From ref. 35. (B) Split-screen format of seven tryptic maps of human transferrin using a narrow-bore column at 0.3 ml/min. LC conditions: column: 5-μm Vydac C_{18} (250 × 2.1 mm I.D.); mobile phase: A = 0.056% TFA in acetonitrile–water (80:20), B = 0.06% TFA in water, gradient conditions: 2–37% A, in 63 min, 37–75% A in 32 min, 75–98% A in 10 min, 0.3 ml/min, 40°C, pressure drop 1800 p.s.i.

Analytical sensitivity in peptide mapping is limited by mobile phase purity and absorbance as well as by detector noise and drift[41]. Fig. 7 shows a high-sensitivity tryptic map of lysozyme at the 15-pmol level using mobile phase system III (phosphate). Note that all peaks are readily identifiable with an acceptable signal to noise ratio at this very low level. A detection limit of about 1 pmol was achieved under these conditions.

Sensitivity is enhanced by using a lower monitoring wavelength (*i.e.*, 210 nm) and by reducing the column diameter. Lowering the monitoring wavelength from 220 nm to 210 nm increases the molar absorptivity of most peptides by a factor of two to

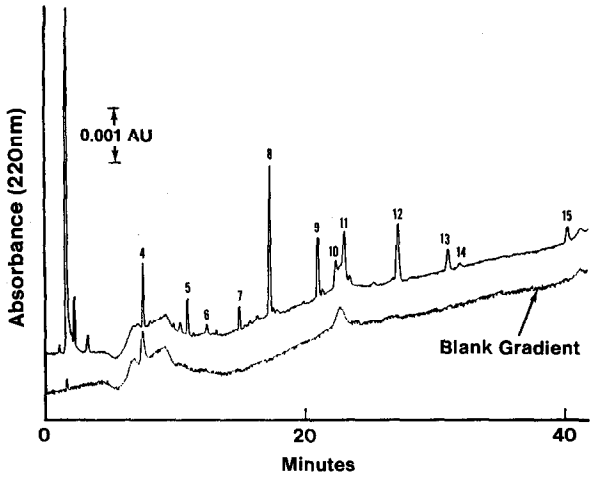

Fig. 7. High-sensitivity tryptic map of 0.2 μg or 15 pmol of lysozyme shown with the elution profile of a blank gradient (data sampling rate was higher for the blank gradient run). LC conditions are identical to those used in Fig. 5 (lower). From ref. 35.

four. However, 0.1% TFA mobile phase (system I) cannot be used effectively at 210 nm due to the increased gradient baseline shifts caused by the unbalanced absorbance. Since phosphate buffer (system III) might be less compatible with commercial sequencers (> 200 μl), the use of the balanced absorbance TFA mobile phase (system II) is recommended for general high-sensitivity mapping[41].

Reducing the column diameter from 4.6 mm to 2.1 mm while maintaining the same linear flow velocity further enhances the mass sensitivity by a factor of five. This is due to the reduction of column void volume which leads to a lower sample dilution. This results in higher peak concentrations in the detector flow cell[42]. Fig. 8 is a tryptic map of 60 pmol of human transferrin using a narrow-bore Vydac C_{18} column (250 mm $\times$ 2.1 mm I.D.) at 0.3 ml/min. The mobile phase system II was used here at 210 nm. Substantial increases in mass sensitivity are apparent when the peak heights are compared with those found in Fig. 3 (after normalization). Any remaining gradient shift can be eliminated by making fine adjustments with TFA[41].

Sensitivity can be further enhanced by using 1-mm microbore columns[41].

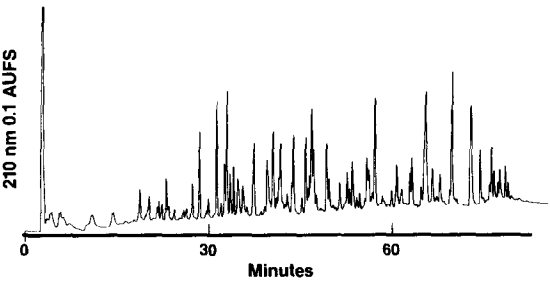

Fig. 8. High-sensitivity tryptic map of human transferrin (60 pmol) using narrow-bore column. Column and mobile phase conditions are identical to those used in Fig. 6B. From ref. 35.

However, their resolution performance might be limited by the column packing technique and also severely hampered by the extracolumn band broadening effects from the instrument[42].

Lowering the flow-rate while maintaining t_G can dramatically increase detection sensitivity since retentions ($\bar{k}$) are reduced (see eqn. 1). Although the peak capacity is adversely affected (see Fig. 4), the number of detected peaks which can be collected might not be substantially reduced[41]. This approach is particularly useful for collection runs because the decrease of peak volumes facilitates direct applications to commercial sequencers.

Analysis speed

Fast separation is desirable to increase productivity in quality control labs if other performance characteristics are not sacrificed (*e.g.*, resolution, sensitivity, reproducibility, column lifetime)[23,38]. This can be achieved by using shorter columns packed with small particles (2–3 μm) operated at faster flow-rates. Fig. 9 shows the Van Deemter curves of columns packed with 10-, 5- and 3-μm particles for peptides. 3-μm particles display lower minimum plate heights (*i.e.*, more plates per unit length) and less resistance to mass transfer (or lower C term, *i.e.*, less efficiency loss at high flows)[32]. The use of elevated column temperatures (40–50°C) enhances performance of fast separations by increasing peptide diffusivities[37,43].

By using an 8 cm long column packed with 3-μm porous C_8 materials at high flow-rates (3 ml/min) and short t_G, tryptic maps of small proteins can be generated in 3–4 min (Fig. 10) . These columns generate very high resolution maps at low flow (1 ml/min) and longer t_G (20–60 min)[38]. Fast peptide analysis is particularly well suited for checking peptide identity and purity from collected fractions.

The elimination of intraparticular diffusion in non-porous particles constitutes a novel approach to fast peptide mapping as demonstrated by Kalghatgi and Horváth

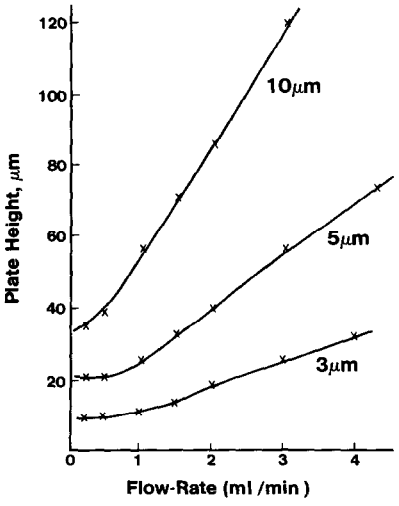

Fig. 9. Van Deemter curves for 10-, 5- and 3-μm particle columns. Mobile phase: acetonitrile–0.1% TFA (25:75), 40°C; sample: met-enkephalin.

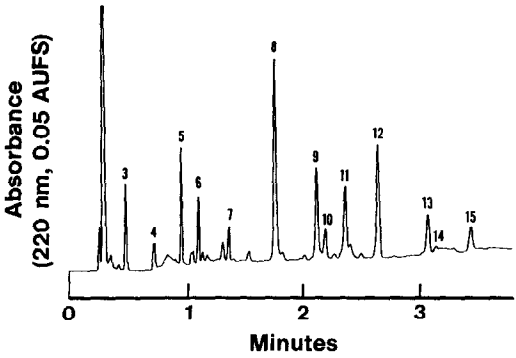

Fig. 10. Fast peptide mapping using an experimental 3-μm porous C_8 bonded phase column. LC conditions: experimental fast peptide mapping column packed with 3-μm porous C_8 bonded phase particles (83 × 4.6 mm I.D.); mobile phase: A = acetonitrile, B = 50 m*M* phosphate buffer (pH = 2.6); Gradient conditions: 0–15% A in 0.8 min, 15–30% A in 3 min, 3.0 ml/min at 40°C, pressure drop = 3200 p.s.i. Sample is a 50-pmol tryptic digest of lysozyme. Peak numbers refer to tryptic fragments of lysozyme in order of elution. Injection was coincidental with the onset of the gradient. From ref. 35.

(Fig. 11)[16]. A very complex tryptic map of rt-PA is achieved in 16 min compared with an analysis time of 100 min (see Fig. 1) on conventional columns.

Peptide identification techniques

Traditional methods of peptide identification by amino acid analysis or sequence analysis can be supplemented by on-line detection techniques. Fig. 12 shows a dual-wavelength tryptic map of protein A. The 220-nm map (which monitors all peptide bonds) is very complex but the 280-nm map is more selective because only peptides containing the aromatic amino acids, phenylalanine, tryptophan and tyrosine, are detected. Typical diode array UV spectra of non-aromatic and aromatic peptides are also shown in Fig. 12. The non-aromatic peptide is not present in the 280-nm map due to a lack of absorbance at that wavelength.

Dual-wavelength detection is a powerful means for peptide identification. Research in spectral deconvolution techniques migh eventually lead to an improved

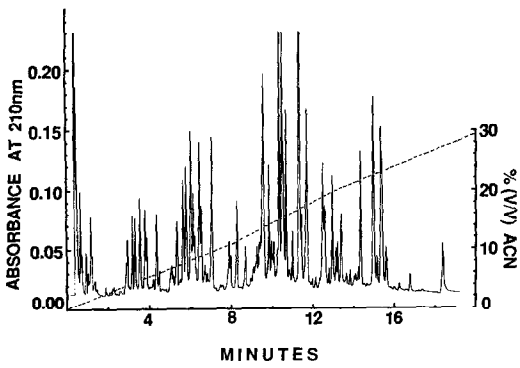

Fig. 11. Tryptic map of rt-PA using an experimental 2-μm micropellicular C_{18} column (75 × 4.6 mm I.D.). Flow-rate 1.5 ml/min, temperature 80°C; ACN = acetonitrile. Courtesy of Kalghatgi and Horváth, from ref. 16.

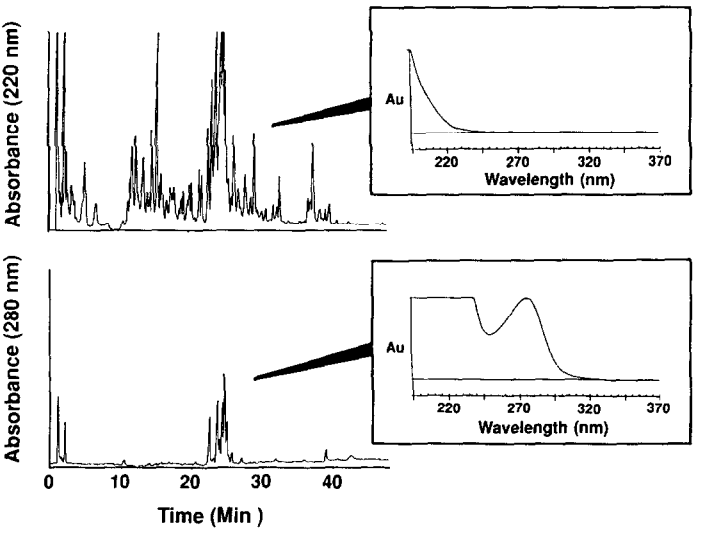

Fig. 12. Tryptic maps of protein A using dual-wavelength detection. Column and mobile phase identical to Fig. 5 (upper).

identification method of aromatic peptides[44]. In addition, coupling the UV detector with a fluorescence[4] or electrochemical detector[11] offers very selective detection for peptides containing tryptophan or cysteine residues.

SUMMARY OF PARAMETERS FOR PEPTIDE MAPPING BY RP-HPLC

Table II summarizes some recommended guidelines on column parameters, operating variables and instrumental requirements for peptide mapping.

TABLE II

GUIDELINES FOR PEPTIDE MAPPING

Column parameters	
Packings	3–5 μm C_8 or C_{18} bonded phase
	125–300 Å pores
Dimension	100–250 mm long
	1–2 mm I.D. for high-sensitivity
Operating variables	
Mobile phase	Strong solvent: 0.052% TFA in acetonitrile–water (80:20)
	Weak solvent : 0.06% TFA in water
Flow-rates	0.5–2 ml/min for 4.6 mm columns
	0.2–0.4 ml/min for narrow-bore columns
Gradient time	30–120 min
Column temperature	Ambient to 50°C
Instrumental requirements	
Pump	Excellent flow and composition precision
	Low pulsation and gradient delay volume
	Solvent degassing and pressurization
Detector	Low dispersion and long pathlength
	Low noise and drift
	Dual-channel (210 and 280 nm)

High-resolution mapping is achieved by using column 150–250 mm long, packed with 5-μm low-silanophilic materials operating at low flow-rate and long gradient time. Reproducibility is achieved with precise LC pumps and stable analytical columns protected by precolumns. High sensitivity is achieved with sensitive detectors operating at 210 nm and by using the balanced absorbance TFA mobile phase. Narrow-bore columns are used to further maximize sensitivity. Fast peptide mapping is achieved by using short columns packed with 3-μm porous or 2-μm non-porous materials. Dual-wavelength and selective detection techniques are effective for quick peptide identification.

ACKNOWLEDGEMENTS

The authors thank F. Vandemark, E. Katz and T. Dion of Perkin-Elmer, C. duMee of Genentech and K. Kalghatgi, K. Stone and K. Williams of Yale University for previewing the manuscript. We also thank R. Garnick of Genentech and K. Kalghatgi of Yale University for allowing us to reproduce Fig. 1 and 11 and K. Stone of Yale University for providing us the human transferrin sample digest.

REFERENCES

1 S. Borman, *Anal. Chem.*, 59 (1987) 969.
2 W. A. Schroeder, in W. S. Hancock (Editor), *CRC Handbook of the Separation of Amino Acids, Peptides and Proteins*, Vol. II, CRC Press, Boca Raton, FL, 1984, pp. 283–300.
3 F. Regnier, *LC · GC, Mag. Liq. Gas Chromatogr.*, 5 (1988) 393 and 473.
4 P. A. Hartman, J. D. Stodola, G. C. Harbour and J. G. Hoogerheide, *J. Chromatogr.*, 360 (1986) 385.
5 W. S. Hancock, *Chromatogr. Forum*, 2 (1986) 57.
6 R. L. Garnick, N. J. Solli and P. A. Papa, *Anal. Chem.*, 60 (1988) 2546.
7 R. C. Chloupek, R. J. Harris, C. K. Leonard, R. G. Keck, B. A. Keyt, M. W. Spellman, A. J. S. Jones and W. S. Hancock, *J. Chromatogr.*, 463 (1989) 375.
8 J. L. Brown and W. K. Robert, *J. Biol. Chem.*, 251 (1976) 1009.
9 L. Varady, K. Kalghatgi and Cs. Horváth, *J. Chromatogr.*, 458 (1989) 207.
10 J. J. L'Italien, *J. Chromatogr.*, 359 (1986) 712.
11 C. Lazure, J. Rochemont, N. G. Seidah and M. Chrétien, *J. Chromatogr.*, 326 (1985) 339.
12 M. W. Dong, J. R. Gant and P. Perrone, *LC, Liq. Chromatogr. HPLC Mag.*, 3 (1985) 786.
13 K. L. Stone and K. R. Wiliams, *J. Chromatogr.*, 359 (1986) 203.
14 K. D. Nugent, E. G. Burton, T. K. Slaterry, B. F. Johnson and L. R. Snyder, *J. Chromatogr.*, 443 (1988) 381.
15 A. M. Crestfield, S. Moore and W. H. Stein, *J. Biol. Chem.*, 238 (1963) 622.
16 K. Kalghatgi and Cs. Horváth, *J. Chromatogr.*, 443 (1988) 343.
17 K. L. Stone, M. B. LoPresti and K. R. Williams, in C. Fini (Editor), *Focus on Laboratory Methodology in Biochemistry*, CRC Press, Boca Raton, FL, 1989, in press.
18 A. J. Banes, G. W. Link and L. R. Snyder, *J. Chromatogr.*, 326 (1985) 419.
19 L. R. Snyder and M. A. Stadalius, in Cs. Horváth (Editor), *High Performance Liquid Chromatography —Advances and Perspectives*, Vol. 4, Academic Press, New York, 1986, pp. 195–309.
20 N. H. C. Cooke, B. G. Archer, M. J. O'Hare, E. C. Nice and M. Capp, *J. Chromatogr.*, 255 (1983) 115.
21 J. D. Pearson and F. E. Regnier, *J. Liq. Chromatogr.*, 6 (1983) 497.
22 E. G. Burton, K. D. Nugent, T. K. Slattery, B. R. Summes and L. R. Snyder, *J. Chromatogr.*, 443 (1988) 363.
23 M. W. Dong, J. R. Gant and B. Larsen, *BioChromatography*, 4 (1989) 19.
24 J. Köhler and J. J. Kirkland, *J. Chromatogr.*, 385 (1987) 125.
25 J. D. Pearson, N. T. Lin and F. E. Regnier, *Anal. Biochem.*, 124 (1982) 217.
26 N. D. Danielson and J. J. Kirkland, *Anal. Chem.*, 59 (1987) 2501.
27 C. T. Mant and R. S. Hodges, *Chromatographia*, 24 (1987) 805.

28 B. F. D. Ghrist and L. R. Snyder, *J. Chromatogr.*, 459 (1989) 25.
29 M. A. Stadalius, M. A. Quarry and L. R. Snyder, *J. Chromatogr.*, 327 (1988) 93.
30 C. T. Mant, T. W. L. Burke, J. A. Black and R. S. Hodges, *J. Chromatogr.*, 458 (1989) 193.
31 R. S. Hodges, J. M. R. Parker, C. T. Mant and R. R. Sharma, *J. Chromatogr.*, 458 (1989) 147.
32 G. E. Deibler, L. F. Boyd, R. E. Martenson and M. W. Kies, *J. Chromatogr.*, 326 (1985) 433.
33 C. T. Mant and R. S. Hodges, *J. Chromatogr.*, 326 (1985) 349.
34 N. Takahashi, N. Isioka, Y. Takahashi and F. W. Putnam, *J. Chromatogr.*, 326 (1985) 407.
35 M. W. Dong, J. R. Gant and F. L. Vandemark, *Am. Biotechnol. Lab.*, 716 (1989) 10.
36 E. Hoff, *LC · GC*, 7 (1989) 320.
37 M. W. Dong, unpublished results.
38 M. W. Dong and J. R. Gant, *LC, Mag. Liq. Chromatogr. HPLC*, 2 (1984) 294.
39 J. L. Glajch, J. J. Kirkland and K. Kohler, *J. Chromatogr.*, 384 (1987) 81.
40 M. W. Hunkapillar, in A. J. Bhown (Editor), *Proteins/Peptide Sequence Analysis, Current Methodologies*, CRC Press, Boca Raton, FL, 1988, p. 87.
41 K. L. Stone, M. B. LoPresti, J. M. Crawford, R. DeAngelis and K. R. Williams, in R. S. Hodges (Editor), *HPLC of Peptides and Proteins: Separation, Analysis and Conformation*, CRC Press, Boca Raton, FL, 1989, in press.
42 R. P. W. Scott, *Small Bore Liquid Chromatography Columns*, Wiley, New York, 1984.
43 F. D. Antia and Cs. Horváth, *J. Chromatogr.*, 435 (1988) 1.
44 L. Servillo, G. Colonna, C. Balestrieri, R. Ragone and I. Gaetano, *Anal. Biochem.*, 126 (1982) 251.