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Analysis of peptide mixtures by capillary high performance liquid chromatography: A practical guide to small-scale separations

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Abstract

Capillary HPLC is a very effective means of separating small amounts of peptides and proteins. Capillary columns ranging from 0.01 mm to 0.5 mm in diameter can be constructed using recycled supports and inexpensive fused silica capillary tubing. Commercial pumping systems and UV detectors can be readily converted for operation in the flow rate range of 0.5–50 μL/min. Detailed procedures are given for the construction of columns and UV detector flow cells. A mixture of peptides derived from the endo Lys C digest of horse heart cytochrome c was used to illustrate various aspects of capillary chromatography of peptides and compare the performance of various-sized capillary columns and UV detector flow cell types.

Keywords: capillary HPLC; mass spectrometry; peptides; UV detection

As the need to do structure analysis on rare peptides and proteins has grown, the search for smaller scale separation techniques has intensified. Capillary electrophoresis (CE) has demonstrated impressive sensitivities and separation efficiencies (Kuhr, 1990). Unfortunately, CE requires a relatively concentrated sample solution of which only a small fraction can be loaded on the column. Consequently, although a sample volume of a few tens of nanoliters can be analyzed, it must be taken from a volume of a microliter or more. Dilute sample solutions are particularly problematic. Some progress has been made along the lines of concentrating the sample within the CE column, but only a 10–20-fold concentration can be achieved (Burgi & Chien, 1991).

In contrast, capillary high performance liquid chromatography (HPLC) using reverse-phase supports has the inherent capability to concentrate very dilute solutions of peptides and proteins. Samples can be loaded from an almost unlimited volume of a predominantly aqueous solution and then be eluted as highly concentrated bands using an increasing gradient of organic solvent. The potential advantages for using increasingly smaller columns for HPLC separations have long been recognized (Novotny, 1981, 1988). There are a large number of scholarly works that have established a sound theoretical and experimental basis of the scope and limitations for microscale HPLC. For recent reviews, the reader is referred to volumes by Ishii (1988) and Yang (1989). Progress is such that 2.1-mm-diameter columns are now the standard for many applications. The routine use of still smaller diameter columns is hampered by the limited availability of prepacked columns and instrumentation suitable for operation at very low flow rates (0.5–50 μL/min). The inadequacies of commercially available pumping systems and UV detectors are particularly acute for gradient operation. The literature on the use of capillary (diameters <1 mm) HPLC for the analysis of peptide and protein mixtures is still rather limited (Cobb & Novotny, 1989; Coutant et al., 1990; Hemling et al., 1990; Deterding et al., 1991a,b; Kassel et al., 1991). In most instances, the driving force has been the desire to interface HPLC separations with mass spectrometry.

Our interest in microscale HPLC is derived from the need to do primary structure analysis on peptides and proteins that are available in limited amounts. Reverse-phase HPLC separations have become indispensable for this work, and as sample amounts decrease, the sample losses and limits of sensitivity associated with normal scale chromatography become increasingly unacceptable. Packed reverse-phase capillary columns with internal di-

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ameters between 0.5 and 0.1 mm are now routinely used in our laboratory for the separation of peptide and protein samples. The transition from normal scale to capillary columns was a major technological advance in our work that was achieved with a minimal amount of expense. The modification of existing pumping systems and UV detectors for low flow operation is relatively easy and inexpensive. Capillary columns, although very costly from commercial sources, can be constructed from recycled supports and low cost fused silica tubing using very simple procedures. The performance of such "homebuilt" columns yields separations that are comparable to those obtained with larger format commercial columns and they can be tailor made to a specific application. We have written this report in the belief that our experience with capillary HPLC will be beneficial to others in the field. In addition to providing detailed descriptions on the construction of columns and UV detector flow cells, we offer a number of comparisons that serve to illustrate various aspects of capillary HPLC design and operation. These studies utilize a mixture of peptides generated by an endoprotease digestion of a readily available protein.

Results and discussion

Peptide digest mixture

The mixture of peptides that results from the digestion of horse heart cytochrome c by endo Lys C (Lee & Shively, 1990) was found to be a convenient test sample for evaluating the performance of various capillary HPLC components. The digest yields 13 peptides of five or more residues that appear as prominent peaks in most HPLC separations (Table 1). Three of these (peaks 3, 9, and 13 in Table 1) are the result of incomplete digestion. Peptides corresponding to peaks 3 and 9 are almost always present. The separation of these lysine-extended peptides from other components in the mixture is very dependent on conditions of the HPLC separation. The amount of peak 13 varies greatly from digest to digest and is generally well separated from its closest neighbor, peak 12. Data included in this report were acquired over a period of several months using a number of different digests. For a given comparison, a single sample was used. The peptide labeled peak 1 is very hydrophilic and is easily lost if a large volume of sample is loaded even at a low percentage of acetonitrile. In addition to the peptides listed in Table 1, there are a number of smaller peptides all less than four residues in length and with masses below 500 u that are ignored for the purposes of this study.

Table 1. *Sequence and molecular weights of peptides observed in the capillary HPLC separations and mass spectral analysis of the mixture from the endo Lys C digest of cytochrome c*

Peak[a]	Residues	Sequence	Molecular weight
1	1-5	GDVEK	588.6
2	74-79	YIPGTK	677.8
3	73-79	YIPGTKK	805.8
4	9-13	IFVQK	633.8
5	40-53	TGQAPGFTYTDANK	1,470.6
6	56-60	GITWK	603.7
7	28-39	TGPNLHGLFGRK	1,296.5
8	80-86	MIFAGIK	779.0
9	89-100	TEREDLIAYLKK	1,478.7
10	89-99	TEREDLIAYLK	1,350.5
11	14-22	CAQCHTVEK + heme	1,632.8
12	61-72	EETLMEYLENPK	1,495.7
13	56-72	GITWKEETLMEYLENPK	2,081.4

[a] Refers to labels in the various chromatograms.

Solvent delivery system

The Brownlee microgradient dual syringe pump used in these studies was designed to provide pulse free solvent gradients at flow rates suitable for 2.1-mm-diameter columns (0.1-0.2 mL/min). At the end of each programmed run, the syringes refill and pressurize to a target pressure and composition. This pressurization step is necessary to rapidly achieve and maintain accurate and stable flow rates. During the pressurization step, there is a net flow of solvent toward one of the syringe pumps due to differences in solvent compressibility between the two buffer solutions. The volume of the lines connecting the two reservoirs to the mixing tee must be sufficiently large to allow for this flow without contaminating one syringe with buffer solution from the other syringe. The delay between the start of a gradient program and the arrival of the gradient at the column is dependent on the dead volume of the lines, dynamic mixing chamber, sample injection valve, and the extent that solvent from one syringe is still present in the line between the other syringe and the mixing tee. For the system used in these studies, the delay in delivering the gradient to the head of the column is 3-4 min at flows of approximately 0.1 mL/min. The time delay in the start of the gradient is inversely proportional to the flow rate and is unacceptably long in the range of flow rates used in this study.

To achieve flow rates in the range of 0.5-20 μL/min, the flow from the pump was divided at a point in the line between the dynamic mixer and the sample injection valve (Fig. 1). The split ratio, which is dependent to some extent on the resistance or back pressure of the two solvent pathways, can vary with changes in temperature and solvent composition. With the pumping system used in these studies, excess flow was diverted through two 1-mm × 25-cm columns packed with 5-μm C18 reverse-phase support. Using this arrangement, the volume of each solvent path roughly corresponds to the split ratio. An exact match between the relative volumes of the cap-

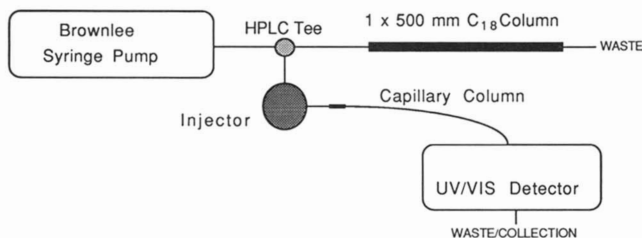

Fig. 1. Diagram of the capillary HPLC system.

illary column and the 1-mm column for the waste flow is difficult to achieve experimentally. Without an exact match, the solvent composition and correspondingly the solvent viscosity in each column will be different during the course of the gradient. As a consequence of these differences, the split ratio will vary somewhat during the course of a run. In a specific instance (Fig. 2A) for a capillary column 0.5 mm in diameter with a gradient run from 2% to 50% acetonitrile in water, the measured flow rate was 18.9 μL/min (2,500 psi) at the beginning, dropped to 18.4 μL/min (2,750 psi) at approximately

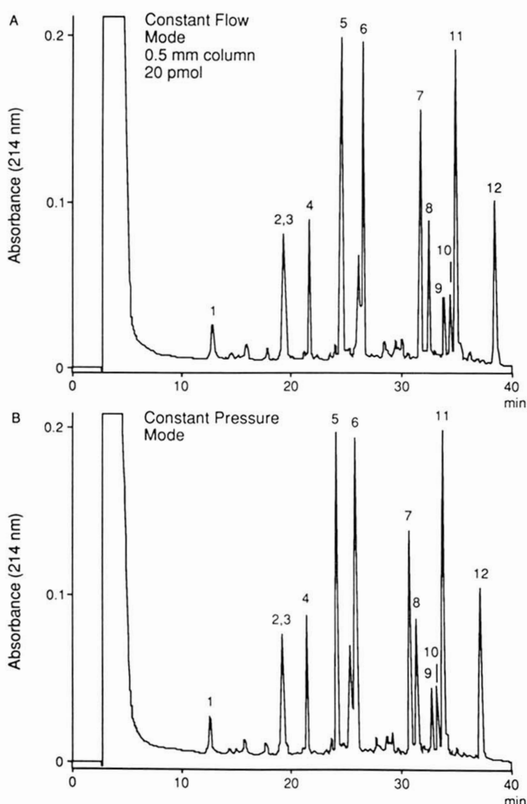

Fig. 2. Comparison of the separation of the endo Lys C digest of cytochrome c on a C18 reverse-phase 0.5-mm-ID packed FSC column using constant flow (**A**) and constant pressure (**B**) modes. Labels over the peaks correspond to entries in Table 1. The gradient was from 2 to 62% acetonitrile over 60 min. The TFA concentration of the aqueous buffer was 0.1% and that of the acetonitrile buffer was 0.085%.

25% acetonitrile, and came back up to 18.9 μL/min (2,690 psi) at 40% acetonitrile. The 2–3% variation in flow rate does not significantly affect the separation of peptide mixtures and is reproducible from run to run.

For smaller diameter columns (0.1–0.25 mm internal diameter [ID]) flow rate variations tend to be larger than for the 0.5-mm-ID columns. More stable operation was achieved by operating in a constant pressure mode. This was accomplished by setting an upper pressure limit on the Brownlee pump to a value somewhat lower than the lowest pressure obtained at a given flow rate setting. With constant pressure maintained at the splint junction, the split ratio is relatively constant. Solvent viscosity varies with composition during the course of the gradient, and consequently the flow rate through the system will also change during the course of the gradient. In a run equivalent to the example given for constant flow operation (Fig. 2B), the flow varied from 18.7 μL/min at the beginning of the run, dropped to 16.8 μL for approximately 20% acetonitrile, and rose to 17.6 μL at 40% acetonitrile. By programming the pressure limit to change during the gradient, it is possible to achieve very nearly constant flow conditions. However, the pressure program must be determined empirically and is not worth the trouble for most applications given the minor but reproducible variations in the flow when operating in the constant pressure mode. The chromatograms for equivalent runs will vary somewhat for the two modes of operation (Fig. 2) because flow rate changes occur in different regions of the gradient program. Both modes have good run-to-run reproducibility and are suitable for the separation of peptide mixtures.

UV absorbance detection and flow cells

The most universal method of monitoring HPLC chromatograms is by measuring the UV absorbance of the solvent eluting from the column. Sensitivity for a given sample component is related to the concentration of the sample peak, the pathlength of the detector cell, and the wavelength of the UV light. For peptides separated on a 0.5-mm-ID packed capillary column, peak volumes are typically in the range of 5–15 μL. For a 0.1-mm-ID column, peak volumes can be as low as 0.3-0.5 μL. Although the 100–1,000 times increase in the concentration of eluted peaks is an obvious advantage for capillary HPLC, there are a number of problems. The volumes of normal scale UV flow cells are too large to maintain the resolution of the separation at these lower flow rates. This extra column variance due to the flow cell is the result of mixing in the void volume of the cell. If the ratio of sample peak volume to the volume of the flow cell is 10:1 or greater, the effect on chromatographic resolution is insignificant (Kirkland et al., 1977). Thus, flow cell volumes need to be in the range of 30–1000 nL depending on the size of the capillary column used. Refractive index effects,

which are a minor concern for conventional flow cells, can pose serious problems for low volume flow cells. Additionally, cell pathlengths for low volume flow cells must be shortened in order to maintain good optical transmittance (Saito et al., 1988). Shorter pathlengths negate part of the sensitivity gains made by having more concentrated peaks.

As part of this study, we explored the use of a commercially available 600-nL (3-mm pathlength) analytical HPLC flow cell. After modifications (see Materials and methods) to reduce postcolumn variance due to the volume of the inlet and outlet lines, the cell was ideally suited for use with 0.5-mm-ID packed columns. The optimal wavelength was 214 nm. Although there is an approximately fourfold increase in signal intensity at 200 nm, the baseline drift due to refractive index changes during gradient runs was excessive (Fig. 3, note the difference in vertical scale between the graphs for 214 and 200 nm). Even at 214 nm, baseline drift is problematic at higher detector sensitivity settings. For a water:acetonitrile:trifluoroacetic acid (TFA) buffer system, the refractive index effects can be minimized by adjusting the concentration of TFA in the two buffer solutions used to form the gradient. If the concentration of TFA in the aqueous component is held constant at 0.1%, baseline drift is minimized if the organic component (acetonitrile:water, 10:1) TFA concentration is between 0.08 and 0.09% (Fig. 3). This is particularly true for gradients between 0 and 60% acetonitrile, which are suitable for the majority of peptide separations. When working with very hydrophobic samples that elute at organic concentrations greater than 60%, better baselines would be achieved by increasing the percentage of TFA to 0.1%. Similar adjustments have to be made in the TFA concentration using the other flow cell, configurations described below. However, refractive index effects are very different for each type of flow cell, and some experimentation was needed to find the best combination of detector wavelength and TFA concentrations to produce good baselines at high sensitivity settings.

A number of flow cells constructed from fused silica capillary (FSC) tubing have been tested (Fig. 4). In the simplest case, the polyimide coating was removed from a section of the transfer line to create a UV transparent window (Fig. 4A). A holder was used to position the capillary in the light beam. An aperture served to restrict the beam to the dimensions of the internal diameter of the capillary. Exact positioning of the capillary with respect to the aperture was needed to avoid severe refractive index effects (data not shown). A commercially available holder with a ball lens (Fig. 4B) was also used. There was essentially no difference between the performance of the holder with the aperture and that using the lens. However, the capillary was much easier to properly position in the holder with the lens.

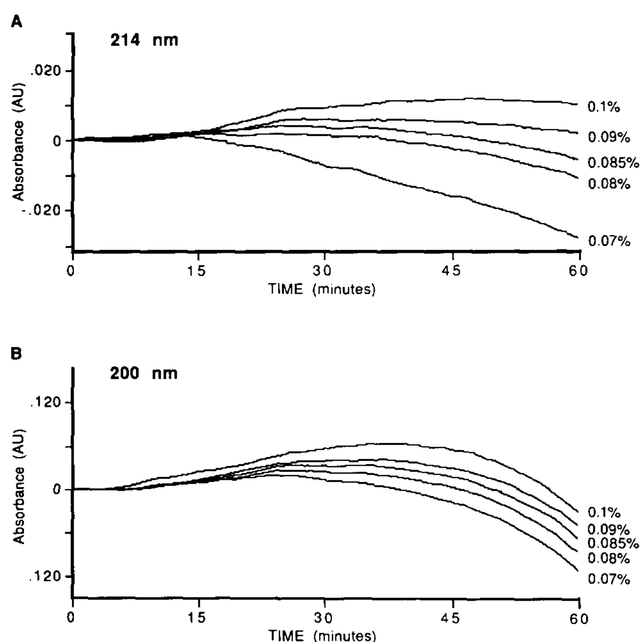

Fig. 3. Effect of TFA concentration in the organic buffer component on the UV detector baseline at 214 nm (**A**) and 200 nm (**B**). The TFA concentration of the aqueous component of the gradient was kept constant at 0.1%. A Shimadzu "Analytical" flow cell (3-mm pathlength, 0.6-μL volume) was used for this comparison with a flow rate of 22 μL/min.

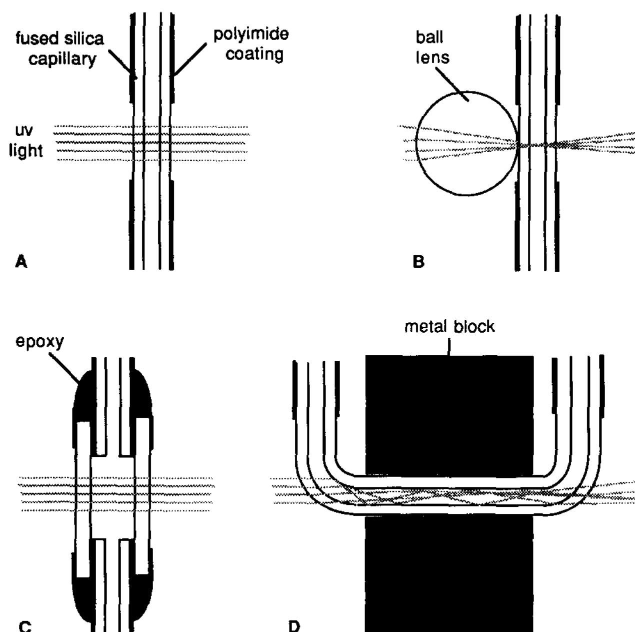

Fig. 4. UV detector flow cells constructed from FSC tubing. **A**: Linear cell with an aperture-limited light beam crossing at right angles. **B**: Use of a ball lens to focus the light through the center of the cell. **C**: Expanded diameter capillary flow cell provides for a greater pathlength. **D**: U-shaped flow cell with the light beam on axis with the capillary.

By expanding the internal diameter of the capillary used as the flow cell (Fig. 4C), it is possible to significantly increase the pathlength and resulting sensitivity. It is convenient in terms of column and flow cell construction to make the flow cell the same diameter as the column. By keeping the gap between the two transfer lines to a minimum, the loss of resolution due to mixing in the cell is negligible as determined from experiments with two flow cells in tandem connected with 50-μm-ID FSC (data not shown).

An alternative method of increasing the flow cell pathlength is to bend a portion of the capillary so that it is parallel to the light beam (Fig. 4D). The use of longitudinal (Z-shaped or U-shaped) capillary UV detector flow cells for capillary liquid chromatography (LC) and CE has been described by Chervet and coworkers (Chervet et al., 1989, 1991). Under ideal circumstances, enhancements in the signal-to-noise ratio of up to 100-fold can be achieved over that obtained with simple perpendicular (on-column) capillary flow cells (Chervet et al., 1989). As part of this study, we have investigated the use of such flow cells for analysis of peptide mixtures. Unfortunately, when working at 200 nm with a TFA, water, and acetonitrile buffer system, there is very little advantage to using the U-shaped cells other than their commercial availability. The commercial U-shaped flow cell used in the comparisons described below had a 0.075-mm ID and 8-mm pathlength. Optimal performance is achieved at flow rates in excess of 4 μL/min in order to minimize loss of resolution due to diffusion.

Using a 0.25-mm-ID packed capillary column and a flow rate of 4 μL/min, the performance of the U-shaped flow cell was very close to that of a 0.25-mm on-column flow cell (data not shown). The signal-to-noise ratio was comparable, and there was only a small loss in resolution using the U-shaped cell. The third factor, which was not very obvious in this comparison, is the limited dynamic range of the U-shaped flow cell. Using the standard TFA buffer system and a 200-nm detector wavelength setting, the signal from the U-shaped cell was close to saturation. A majority of the light reaching the detector photodiode was transmitted through the walls of the capillary. As a consequence, there was a nonlinear response with respect to the amount of peptide in a given peak. The problem is clearly illustrated using the U-shaped flow cell with a 0.16-mm-ID column at a flow rate of 1.5 μL/min (Fig. 5). At a level of 10 pmol, chromatograms obtained using the on-column and U-shaped flow cells are very similar although there is a noticeable difference in relative peak heights. Using a sample level of 50 pmol, signals from the U-shaped flow cell are clearly saturated. Although not quite linear, the signal intensity of the on-column flow cell more accurately reflects the amount of sample present.

Attempts were made to construct U-shaped flow cells in our laboratory that would be more suitable for the

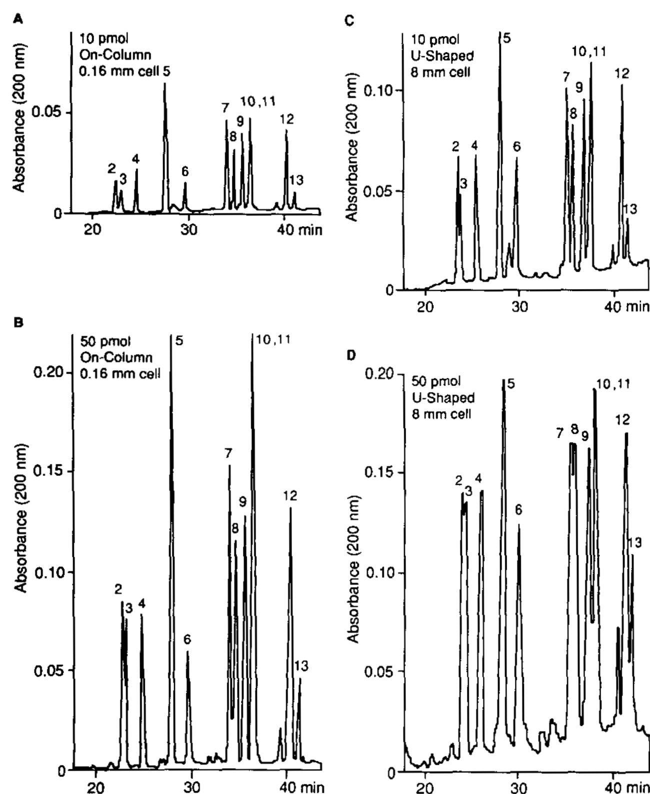

Fig. 5. Comparison of the HPLC analysis of the Lys C digest mixture of cytochrome c using (A) a 0.16-mm-ID on-column flow cell and 10 pmol of sample, (B) a 0.16-mm-flow cell and 50 pmol of sample, (C) an 8-mm (0.75-mm-ID) U-shaped cell and 10 pmol of sample, and (D) an 8-mm U-shaped flow cell and 50 pmol of sample. A 0.16-mm-ID column was used for the separation with a gradient of 2 to 62% acetonitrile in 60 min at a flow rate of 1.5 μL/min.

smaller-sized columns. We were unable to achieve any improvement over simple on-column detection. Indeed, most of the U-shaped cells we constructed did not perform very well. Alignment with the light beam of the detector is very critical. There was also considerable variation between two identical (dimensions) commercial U-shaped flow cells in terms of signal-to-noise ratios and refractive index effects.

Column performance

The separation of the endo Lys C digest mixture of cytochrome c on 0.5-mm and 0.16-mm-ID capillary columns compares favorably with that obtained using a commercial 2.1-mm column packed with the same support (Fig. 6). In the comparisons shown, the same sample solution was used for each analysis. The gradients used in these runs do not correspond exactly for the reasons described above. No attempt was made to optimize the separations. Consequently, we are unable to state with certainty that the apparent increase in resolution going from the 2.1-mm- (Fig. 6A) to the 0.5-mm- (Fig. 6B)

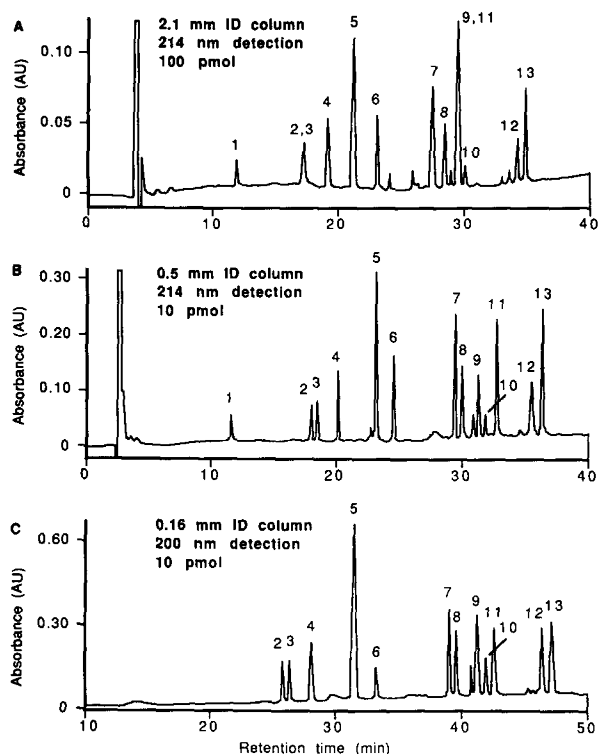

Fig. 6. Comparison of the HPLC separation the Lys C digest mixture of cytochrome c using different diameter columns. **A**: a 2.1-mm column, 100-pmol sample in 10 μL, and a 0.15-mL/min flow rate. **B**: A 0.5-mm column, 10-pmol sample in 10 μL, and a 0.02-mL/min flow rate. **C**: a 0.16-mm column, 10-pmol sample in 10 μL, and a 0.002-mL/min flow rate. In each instance the gradient was 2–62% acetonitrile over 60 min. TFA concentration in the acetonitrile buffer solution was (A) 0.1%, (B) 0.085%, and (C) 0.075%.

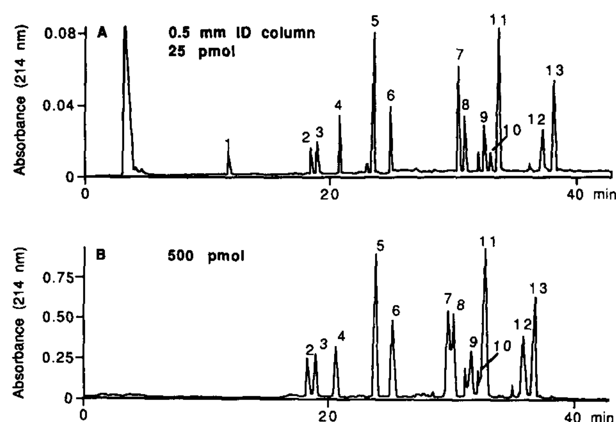

Fig. 7. Comparison of the HPLC analysis of the Lys C digest mixture of cytochrome c on a 0.5-mm-ID column at sample levels of 25 pmol (A) and 500 pmol (B). Sample injections were at a concentration of 10 pmol/μL. Column flow rate was 20 μL/min. Gradient was 2–62% acetonitrile in 60 min.

diameter column is due solely to the difference in column diameter. What we can say with certainty is that in our experience there has never been a compromise in the quality of the chromatography by using capillary size columns. The choice of which column size to use is largely independent of separation efficiency and more a question of the amount of sample to be analyzed and the fate of the sample after the HPLC separation.

For preparative work, the combination of a 0.5-mm-ID column and and the 600-nL Shimadzu flow cell is the system of choice. The flow rate of 15–25 μL/min is convenient for collecting peaks for subsequent analysis by Edman microsequencing, mass spectral (MS) analysis, and amino acid composition analysis. Although sensitivity is sufficient for excellent signal-to-noise ratios at a sample level of 25 pmol (Fig. 7A), sample amounts as large as 500 pmol can be chromatographed with only a marginal loss of resolution (Fig. 7B). Samples elute at concentrations and volumes which eliminate the need to concentrate the collected peaks further for the next analysis.

For very low sample amounts (<10 pmol), the smaller (<0.5-mm) capillaries will have better sensitivities. For the 0.16-mm capillary columns used in this study, good chromatography can be done in the range of 0.5–100 pmol (Fig. 8). Some applications, such as online LC/MS analyses, require very low flow rates for optimal performance. The 2-μL/min flow rate of a 0.16-mm packed column is ideal for the electrospray interface of a quadrupole mass spectrometer.

Tandem UV detection-mass spectrometry

Although online LC/MS analysis does not require the simultaneous use of UV detection, having this capability does serve a number of very useful functions. When problems are encountered with an LC/MS run, it is sometimes difficult to know if the fault lies with the HPLC or the mass spectrometer and interface. Tandem online UV detection provides the means to ensure that the HPLC is operating properly. The chromatogram that is obtained using UV detection can be much different from that obtained by monitoring the total ion current at the mass spectrometer detector. There are always differences in relative peak intensities, and some components in a mixture may not yield a signal with either the UV detector or the mass spectrometer. Consequently, having both means of detection can yield complementary information. Tandem UV detection is particularly useful in correlating LC/MS runs with offline HPLC separations of the same sample. Given the low cost of a UV detector relative to the cost of an LC/MS system ($5,000 vs. $150,000–500,000). It makes good sense to include UV detection, provided the quality of the HPLC separation is not compromised and the system is no more difficult to use.

In the example of an LC/MS run given in Figure 9, the flow from the on-column UV detector cell is transferred

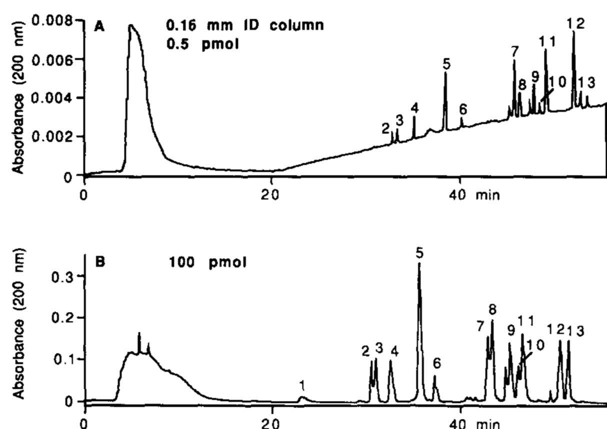

Fig. 8. Comparison of the HPLC analysis of the Lys C digest mixture of cytochrome c on a 0.16-mm-ID column at sample levels of 0.5 pmol (**A**) and 100 pmol (**B**). Sample for 0.5 pmol was loaded at a concentration of 1 pmol/μL. Sample for 100 pmol was loaded at a concentration of 10 pmol/μL. Gradient was 2-62% acetonitrile in 60 min at an initial flow rate of 2 μL/min in the constant pressure mode. TFA concentration in the acetonitrile buffer was 0.075%.

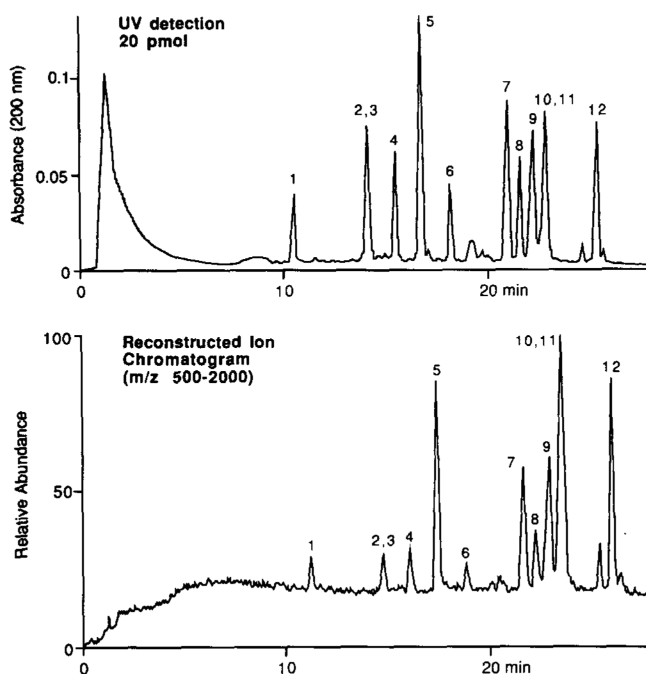

Fig. 9. Online LC/MS analysis of 20 pmol of the Lys C digest mixture of cytochrome c using tandem UV detection. Upper trace is UV detection chromatogram. Lower trace is the chromatogram reconstructed from the total ion current at the mass spectrometer detector. Gradient was 2-62% acetonitrile in 30 min at a flow rate of 2 μL/min.

to the electrospray interface via a 0.025-mm-ID transfer line. There is no discernible difference in chromatographic resolution going from the UV detector to the mass spectrometer. An apparent 35-s delay between the UV detector signal and that of the mass spectrometer is due to the total transit time of sample molecules from the UV cell to the mass spectrometer ion detector. The majority of that time is spent in the electrospray interface.

Conclusions

With minor modifications, commercial HPLC solvent delivery systems and UV detectors can be made compatible with the gradient elution of peptide mixtures using capillary columns with internal diameters ranging from 0.1 to 0.5 mm. Fused silica capillary columns of this size can be constructed using relatively simple methods and packed with standard reverse-phase supports. The performance of packed capillary columns for the separation of the peptide mixture obtained from the endo Lys C digest of cytochrome c is as good as that obtained with commercially available 2.1-mm microbore columns. For routine separations in the range of 10-500 pmol, a 0.5-mm-ID capillary column is the most practical choice. Flow rates are in the range of 15-25 μL/min. Sample peaks are easily collected at concentrations suitable for subsequent microsequencing, mass spectrometry, and amino acid composition analysis. For sample amounts below 10 pmol or for applications that require very low flow rates such as online LC/MS analysis, smaller diameter columns on the order of 0.2 mm are preferred. Simultaneous online UV detection and mass spectral analysis provide a powerful means of characterizing peptide mixtures.

Materials and methods

Solvent delivery system

All chromatographic runs utilized a Brownlee (Applied Biosystems Inc., Foster City, California) Microgradient System equipped with a 0.16-mL dynamic mixer and a Rheodyne (Rheodyne, Inc., Cotati, California) Model 7125 injector. All tubing downstream from the flow selector valve was replaced with 0.01-inch-ID stainless steel tubing to reduce the dead volume between the pump and the injector. A Rheodyne Model 7000 switching valve was installed upstream from the dynamic mixer to facilitate purging of the pumps.

UV detection

Three different UV detectors were used in this study. A Shimadzu (Shimadzu Scientific Instruments, Inc., Columbia, Maryland) Model SPD-6A variable wavelength detector with a standard flow cell (10-mm pathlength, 8-μL volume) was used for work with 2.1-mm columns. For work with 0.5-mm columns, the standard flow cell was replaced with a Shimadzu "Analytical" flow cell (3-mm pathlength, 0.6-μL volume) that was modified as described below. For work with capillary flow cells of the

type shown in Figure 4, either a Kratos (Applied Biosystems, Inc., Ramsey, New Jersey) Spectroflow 757 or an ABI (Applied Biosystems, Inc., Ramsey, New Jersey) Model 759 variable wavelength detector was used. Both were equipped with an ABI Capillary Flowcell Holder, which utilizes a ball lens to focus the light beam through the capillary. The capillary flow cell was properly positioned in the holder by placing the assembly on a light box and observing the light pattern through the capillary with a 10× magnifying lens. A symmetrical pattern is an indication that the flow cell is properly centered, which is important to reduce refractive index effects. Some work was also done with other flow cell holders that utilized apertures to restrict light transmission to the center of the capillary. When properly centered, performance was equivalent to the holder using the ball lens, but alignment was generally more critical and difficult to accomplish. The longitudinal (U-shaped) flow cell (LC Packings, San Francisco, California) had a 0.07-mm ID, 270-mm outer diameter (OD), and a pathlength of 8 mm.

Modification of the Shimadzu analytical flow cell

The Shimadzu 600-nL (3-mm pathlength) analytical flow cell was suitable only for use with the 0.5-mm-ID columns. The flow cell comes with 0.2-mm-ID stainless steel tubing for the cell inlet line and 0.3-mm-ID stainless steel tubing for the outlet line. The inlet line is attached with a standard HPLC fitting and is easily removed. A piece of 1.6-mm-OD (0.4-mm-ID) teflon tubing was placed over the 0.35-mm-OD (0.05-mm-ID) transfer line (approximately 1 cm long) used to retain the frit at the end of a 500-μm column. The transfer line was then attached directly to the cell using a vespel ferrel. With this arrangement, the dead volume from the end of the column to the flow cell is negligible. The outlet line is permanently attached to the cell and not easily replaced. It has a dead volume of 7 μL. Replacement of the outlet line is necessary if peaks are to be collected. The outlet line was shortened to 5 mm, and a 0.25-mm-OD × 0.1-mm-ID FSC tube was inserted into the entire length of line and secured with epoxy. At a distance of 4 cm from the cell, a zero dead volume HPLC union was used to couple the cell to a 0.05-mm-ID fused silica line. Thus, the total dead volume of the outlet line from the cell to the point of collection is approximately 1 μL.

Capillary column and flow cell construction

In this section, a step-by-step procedure is given for the construction of a 0.16-mm-ID packed fused silica column incorporating a capillary UV flow cell and a transfer line used to interface with an electrospray source of a mass spectrometer. Columns of any size ranging from 0.1 to 0.5-mm-ID with a variety of flow cell combinations can be constructed using these same general methods. All FSC tubing is from PolyMicro Technologies, Inc. (Phoenix, Arizona). Where indicated, the polyimide coating on the FSC tubing was removed using an electrically heated wire coil (Stripper, PolyMicro Technologies). A diagram of the completed column and flow cell is given in Figure 10. The vertical scale in the diagram has been exaggerated by a factor of 4–5 in order to enhance the detail of the various components. A 40-cm length of 0.16-mm-ID × 0.35-mm-OD FSC is used as the column body. A layer of either Zytex membrane (extra course grade, Nor-

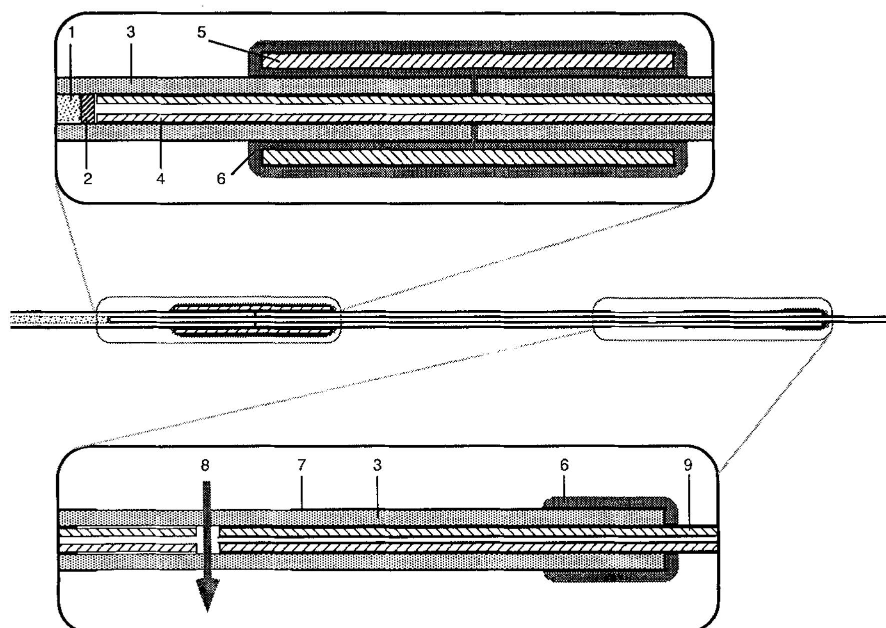

Fig. 10. Schematic showing the construction of a 0.16-mm-ID fused silica column with an integral capillary flow cell. Numbered components are as follows: 1, column packing, 2, glass fiber frit; 3, 0.16-mm-ID × 0.35-mm-OD FSC; 4, 0.05-mm-ID × 0.15-mm-OD FSC; 5, 0.53-mm-ID × 0.72-mm-OD FSC; 6, Epoxy; 7, polyimide coating; 8, UV light beam; 9, 0.025-mm-ID × 0.15-mm-OD FSC transfer line.

ton/Chemplast, Wayne, New Jersey) or Whatman GF/A glass fiber filter paper (Whatman Ltd., Maidstone, England) was used as a frit to retain the column packing. Both are suitable; however, columns using the glass fiber paper have significantly lower back pressure. The Zitex or glass fiber paper is held in place with a length of 0.05-mm-ID × 0.15-mm-OD FSC extending 1–2 cm into the column body. This tube also serves as the transfer line to the UV flow cell. The flow cell is made from a second piece of 0.16 × 0.35-mm-FSC from which the polyimide coating has been removed to create a UV light path. The distance from the frit to the flow cell is typically 5–7 cm. The column, flow cell, and transfer line between the two are held in place with epoxy resin reinforced with a 1-cm length of 0.53-mm-ID x 0.72-mm-OD FSC. A length of 0.025-mm-ID × 0.15-mm-OD FSC forms the other side of the flow cell and serves as a transfer line to the electrospray MS interface. The gap between the two transfer lines that forms the flow cell is approximately 1 mm. Approximately 2 cm of tubing centered at the UV window must be free from epoxy in order to allow for proper positioning of the flow cell in the flow cell holder.

The actual assembly of the column and flow cell requires some practice. The Zitex or glass fiber paper frit is cut from a sheet using the end of the column capillary. The end of the column is pressed against the membrane while backing it with a gloved finger. The result is a circular section that remains on the end of the capillary. The end of the capillary is firmly tamped on a hard surface to seat the central portion of the frit within the capillary. The frit is pushed into the column using the 0.15-mm-OD transfer capillary as a ramrod. The transfer line to the mass spectrometer is generally added to the column after packing in order to facilitate the packing operation.

The gluing operation is diagrammed in Figure 11. The flow cell capillary and reinforcing capillary are placed over the transfer line leaving a gap (Fig. 11A). A small spot of epoxy is placed at the exposed junction (Fig. 11B). The tip of a soldering iron is used to heat the epoxy (without touching it) causing it to flow into the gap between the flow cell FSC and the reinforcing FSC (Fig. 11C). Once the interstitial area is filled with epoxy, the column and flow cell capillaries are pushed together, and the reinforcing capillary is slid into place over the junction. The entire assembly is then thinly coated in epoxy (Fig. 11D). After the epoxy has begun to set, the column is secured in a vertical position, column-side down, and allowed to harden overnight.

For the 0.5-mm-ID columns, the frit is held in place with a 0.5-mm-ID × 0.35-mm-OD transfer line. There is sufficient space between the two capillaries that epoxy is allowed to flow almost to the frit. The frit is positioned a small distance beyond the transfer line during the gluing operation. The frit is forced against the end of the transfer line when the column is packed.

The detector flow cell need not be an integral part of

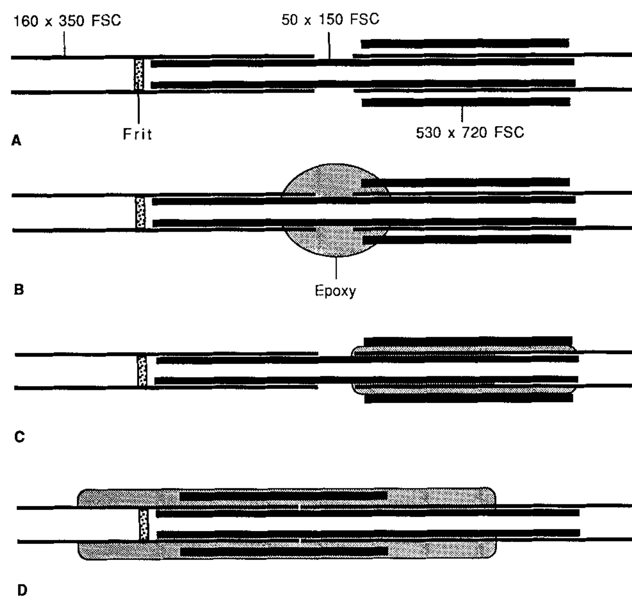

Fig. 11. Diagram illustrating the steps used in constructing fused silica capillary columns. See text for detailed description.

the column. A 1–2-cm length of teflon tubing (0.25-mm-ID, Upchurch Scientific, Inc., Oak Harbor, Washington) can be used to join a 0.35-mm transfer line from a column to the flow cell. The opening is first enlarged with a piece of 0.35-mm-OD FSC. Such a connection can be reused many times and is easily replaced if necessary. It provides an easy means to couple the same column to different flow cells. However, there is the danger that the capillary may slip out of the teflon sleeve due to restricted flow in the transfer line or mechanical strain. A 0.35-mm-OD FSC sleeve can be used to increase the OD of smaller diameter transfer lines.

Column packing

Columns used in this study were packed with Vydac C18 reverse-phase support having a 5-μm average particle size and 300-Å pore size. The packing material was recycled from old commercial Vydac columns, from which the top portion of the column has been discarded. The remainder is washed extensively with acetonitrile. A slurry of the support in acetonitrile (0.34 g/mL) is loaded into a 150 × 2.1-mm stainless steel slurry reservoir with a 10-mL syringe. The slurry reservoir is connected to the capillary column (filled with acetonitrile) with a Valco reducing union ($\frac{1}{16}$ inch to $\frac{1}{32}$ inch) using a graphite capillary ferrule (0.4 mm ID). The slurry reservoir is connected to an HPLC pump in which the pressure has already been brought to 6000 psi against a closed valve between the pump and the slurry reservoir. Once the connections are made, the valve is opened and the column is quickly packed. After the column is completely filled, the reser-

voir is removed and the column is reconnected to the pump for an additional 30 min of packing.

Flow rate measurement

Flow rates were measured at the end of either the column or flow cell transfer line using a graduated glass capillary pipette (5 or 25 μL size depending on the flow rate). The pipette was held in contact with the fused silica tubing. Solvent eluting from the end of the tubing was drawn into the pipette by capillary action. Solvent flow between the graduations was timed with a stopwatch.

HPLC buffers

All gradient separations used a standard TFA buffer system. The TFA concentration in the aqueous buffer solution was always 0.1%. The organic buffer solution was 90% acetonitrile and 10% water. The TFA concentration varied from 0.07% to 0.1% as noted in each figure caption.

Peptide digest mixture

A stock solution of cytochrome c (equine heart: Sigma, St. Louis, Missouri) was prepared at an approximate concentration of 1 mg/mL in Milli-Q-filtered water. The absolute concentration was determined by amino acid analysis. The proteolytic digestion of 2 nmol of cytochrome c by endo Lys C was performed at a 1:100 mole ratio (E:S) in 100 mM Tris, pH 9.0, in a volume of 100 μL for 18 h at 37 °C. The reaction was terminated by the addition of an equal volume of 1% TFA to produce a working concentration of 10 pmol/μL.

Mass spectrometry

LC/MS analyses were obtained using a Finnigan MAT TSQ-700 triple sector quadrupole mass spectrometer equipped with an electrospray ion source. The standard Finnigan multilayered electrospray needle assembly was modified by replacing the innermost metal needle with the 0.025-mm-ID, 0.150-mm-OD transfer line from the UV detector flow cell. The end of the transfer line was retracted a few millimeters into the middle concentric metal needle, which itself protruded a few millimeters beyond the outer metal needle. Flow from the HPLC was 2 μL/min. A sheath fluid consisting of 2-methoxyethanol was flowed at a rate of 2 μL/min through the middle needle, and a flow of nitrogen sheath gas was directed through the outer metal needle. Mass spectra were collected over the range m/z 500–2,000 with a scan-to-scan cycle time of 3 s.

Acknowledgments

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