Low Flow High-Performance Liquid Chromatography Solvent Delivery System Designed for Tandem Capillary Liquid Chromatography–Mass Spectrometry

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A solvent delivery system is described that is designed to increase the efficiency of liquid chromatography–mass spectrometry (LC/MS) analyses. Gradients formed by using two low pressure syringe pumps are stored in a length of narrow bore tubing (gradient loop) mounted on a standard high pressure switching valve. The preformed gradient is pushed through the column by using a high pressure syringe pump. The system is fully automated and can be controlled with either a personal computer or the mass spectrometer data system. Advantages include gradient operation without the use of split flows, pressure programed flow control for rapid sample loading and recycling to initial conditions, and a flow rate range of 0.1–20 μL/min, which is suitable for packed capillary columns 50–500 μm in diameter. The system has been used extensively for rapid molecular weight determinations of intact protein samples, as well as LC/MS and liquid chromatography–tandem mass spectrometry analyses of complex peptide mixtures. *(J Am Soc Mass Spectrom 1995, 6, 571–577)*

Much of the development of capillary column liquid chromatography (LC) in recent years has been driven by the need to couple the separation power of high-performance liquid chromatography (HPLC) to the structure analysis capabilities of mass spectrometry. With the development of continuous flow fast-atom bombardment (flow FAB) [1] and electrospray ionization [2], the interface of liquid chromatography to mass spectrometry has become a practical reality. The vacuum environment of the mass spectrometer places severe restrictions on the volume of liquid that can be introduced into the instrument. For normal scale chromatography (4.6-mm columns, 1-mL/min flow rates), the effluent from the column must be split, with the major fraction (99.5–99.9%) going either to waste or to a fraction collector. With microbore chromatography (2.1-mm columns, 0.2 mL/min), the fraction that goes to waste is correspondingly less (98.5–99.5%). The split is accomplished in different ways dependent on the ionization method. For flow FAB, the split must be made between the column and the flow FAB probe. The same was true for electrospray in its original form. Only 2–10 μL could be delivered to the electrospray needle. With pneumatic [3], ultrasound [4], or thermally [5] assisted nebulization, electrospray sources are able to accommodate flows to the needle up to 1 mL/min. The split is then made in the spray region of the electrospray source with the excess sample and solvent either evaporated or collected as waste. When the chromatography is down-scaled to flow rates suitable for the mass spectrometry, sample and solvent waste can be avoided and the chromatographic resolution that capillary HPLC provides increases.

The benefits of capillary scale separations have long been recognized [6–10]. Suitable columns are easily constructed [11, 12] or can be purchased from commercial sources. Unfortunately, the advent of suitable pumping systems has been slow. Systems specifically designed to deliver low flow rates are much more expensive than standard pumping systems. Many commercial systems are capable of delivery of gradients at flows of a few microliters per minute. Unfortunately, even with the necessary changes in tubing diameters and mixing chambers, the dead volume between the point at which the gradient is formed and the top of the column often results in unacceptable delays in the start of the gradient. The method used in most labs to get around the dead volume problem is to operate the pump at a much higher flow and split most of it to waste prior to the sample injector and

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column. Although split flow operation allows the use of normal scale pumping systems with capillary columns, it is an imperfect solution. Solvent consumption is higher than necessary and care must be taken to ensure that the split ratio does not change during operation. In this report, we describe in detail a low flow solvent delivery system for capillary HPLC in which 100% of the solvent flow is delivered to the column. The system was specifically designed to minimize the turnaround time between liquid chromatography–mass spectrometry (LC/MS) runs. The result is a greatly increased sample throughput compared to our previous pumping system that utilized split flows. The system can be constructed from off the shelf components and is controlled by either a personal computer or the mass spectrometer data system computer. The total cost of system components compares favorably to that of a standard HPLC system.

Experimental Methods

System Components

Solvent delivery system components (Figure 1) include two Harvard Apparatus (South Natick, MA) model 44 microprocessor-controlled syringe pumps that use firmware version 2.3, an Isco, Inc. (Lincoln, NE) model 100 DM high pressure syringe pump that operates under pump controller firmware revision G, four CKD Corp. (Nagoya, Japan) 12 VDC electropneumatic relays, a Rheodyne, Inc. (Cotati, CA) model 7125 manually actuated sample injector, a gradient valve system that consists of a Rheodyne model 7000 six-port two-position valve driven by a Rheodyne model 5701 two-position pneumatic actuator, and a solvent refill system that consists of two Rheodyne model 5301 three-way slider valves with Rheodyne model 5300 pneumatic actuators. Flow from the Harvard syringe pumps was combined via a Valco (Houston, TX) 1/16-in. stainless steel tee with a 0.01-in. bore.

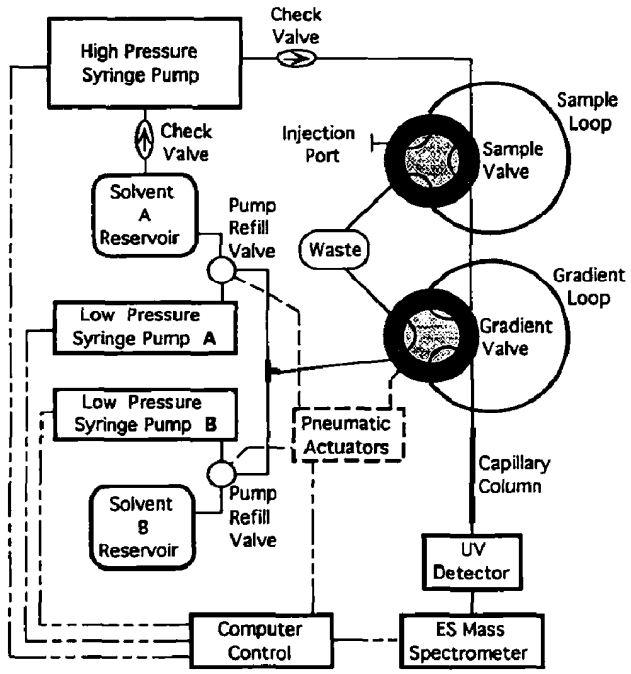

Figure 1. Gradient loop HPLC system for use with capillary columns. The sample valve is shown in the off-line or "load" position. The gradient valve is shown in the on-line position.

Gradient loops were constructed from stainless steel tubing (Alltech Associates, Dearborn, MI) with volumes that ranged from 5 to 1000 μL dependent on the size and flow rate of the column. Suggested gradient loop sizes for various column sizes are given in Table 1. For experiments used to demonstrate retention time reproducibility, somewhat better results were obtained by using a 150-μL gradient loop with 0.01-in. i.d. rather than the 200-μl loop with the 0.02-in. i.d. that we normally use. When retention time reproducibility is not critical, the larger loop provides greater flexibility to form different gradients and is less susceptible to plugging by stray particulate matter in the system. System dead volume was minimized by using 0.007-in. i.d. tubing for transfer lines from the injector to the gradient valve, from the mixing tee to the gradient valve, and from the gradient valve to the column. For columns 200 μm and larger, 0.01-in. tubing can be used for transfer lines between various system components. For work with 200–500-μm i.d. columns, the Harvard syringe pumps used Hamilton (Reno, NV) 250–500-μL gas-tight syringes and the gradient loop was filled at flow rates of 100–150 μL/min. For work with columns 50–100-μm i.d., 25–100-μL Hamilton gas-tight syringes were used and the gradient loop was filled at rates of 25–50 μL/min.

Fused silica capillary (FSC) columns were prepared via methods previously described [11] and were packed with Vydac (Hesperia, CA) C18 reverse phase support. Column flow rates were determined by filling a calibrated glass capillary micropipette (1–5 μL; S/P Accupette, Baxter Diagnostics, McGraw Park, IL) at an intermediate column pressure (1000–2000 lb/in.^{2}). The observed flow rate was used to calculate a column constant that had units of microliters per minute per pound per square inch. For a given column and solvent viscosity, the flow rate is linear with respect to pressure and the column constant is used by the data

Table 1. Suggested sizes for the volumes and internal diameters of gradient loops for use with different column sizes and flow rates

Column		Gradient loop	
Diameter (μm)	Flow rate (μL/min)	Volume (μL)	i.d. (in.)
500	20	1000	0.02
250	2	200	0.02
100	0.5	25–50	0.01
50	0.1	5–10	0.007

system to calculate the pressure required for the user-selected flow rate. Connection to the standard Finnigan-MAT electrospray source was made via a 50-μm i.d., 150-μm o.d. FSC transfer line that extends to the end of the electrospray needle (300-μm i.d., 400-μm o.d.). Unless otherwise noted, a 2-μL/min flow of 2-methoxyethanol was used as a sheath liquid and nitrogen was used as the sheath gas (gauge pressure 60 lb/in.^{2}). Gradient elutions were done as indicated in the figures by using 0.1% aqueous trifluoroacetic acid (TFA) for solvent A and 90% acetonitrile–10% water–0.07% TFA for solvent B. Solvents were degassed with helium and helium was used to activate the pneumatic valves. Column effluent was monitored at 200 nm by an Applied Biosystems Inc. (Foster City, CA) model 759A UV detector. The signal from the UV detector also was monitored with a Soltec Corp. (Sun Valley, CA) model 1241 single pen strip chart recorder.

Computer Control

Harvard syringe pumps are "daisy-chained" together on the same RS232 serial data line and programed individually by a remote computer. Each pump can store a sequence of up to nine scheduled events, which are then executed with a single command. This feature allows complete gradient profiles to be sent to the pumps in advance. When the pumps are instructed to run, their internal microprocessors handle gradient step timing, which leaves the computer free to control valve timing and other events.

An Apple MacIntosh SE30 personal computer originally was used to program the Harvard syringe pumps independently of the mass spectrometer data system. Details of the hardware and software components for this setup are available from the authors. For the work described in this article, the solvent delivery system was controlled by a Finnigan-MAT (San Jose, CA) TSQ 700 mass spectrometer data system that operated under ICIS version 7.2 and Instrument Control Language (ICL) version 7.4. The GUIDE view of the data system was programed to provide a user friendly graphic interface to the LC system. Gradient profile information and UV detector output are displayed in the GRAPH view of the data system (Figure 2). During LC/MS operation, use of the chart recorder is redundant because analog input received from the UV detector is plotted to the GRAPH view in real time and saved with mass spectral information during data acquisition. When the chart recorder is in place it is possible to uncouple the LC from the mass spectrometer and use them as two separate instruments controlled by a single computer. Normally, the nine possible program steps are used as follows: a column wash step that consists of 100% solvent B, back end insurance volume (BEI), up to five linear gradient steps, front end insurance volume (FEI), and a stop command. A separate command sequence is sent to refill the pumps. The ICL programs and hardware configurations used to control system components are available from the authors.

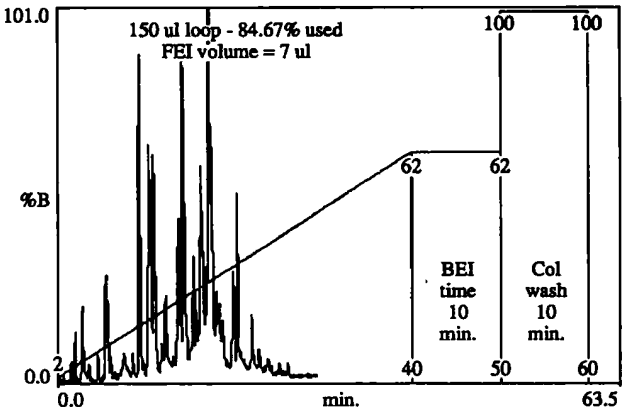

Figure 2. Actual display for a gradient program and UV trace after 30 min in the GRAPH view of the Finnigan-MAT TSQ-700 data system. The display provides information to the user with regard to sample loop size and the percent of the loop used to store the gradient, the front end insurance volume (FEI), the gradient profile, and the back end insurance (BEI) and column wash times. Numbers at different points on the gradient contour indicate the gradient composition in terms of percent solvent B (%B). Numbers near the x axis indicate the time in minutes for each of those points. During the course of the run, the UV detector chromatogram is superimposed on the gradient profile. No absorbance scale is provided for the UV trace. The sample used to generate the UV absorbance profile was a tryptic digest mixture of human hemoglobin.

Results and Discussion

System Design and Principles of Operation

The method of gradient formation is a variation of the procedure described by Ishii et al. [6] in their pioneering work on capillary HPLC. In that work, the gradient was formed prior to the run and stored in a tube that was then brought on-line with the flow through the column. In our design (Figure 1), the gradient is formed by two low pressure syringe pumps that operate under computer control and is stored in a length of stainless steel HPLC tubing (gradient loop) mounted on a pneumatically actuated HPLC valve (gradient valve). Actuation of the valve interposes the gradient loop into the stream of solvent that flows from a high pressure syringe pump to the column. The sequence of events can be described briefly as follows: With the sample loop and gradient loop off-line, solvent A flows from the high pressure syringe pump to the column. Sample solution is injected into the sample loop. The sample loop is brought on-line and sample solution is pushed onto the column. When sample loading is nearly complete, the gradient is formed by two low pressure syringe pumps that push a varying ratio of solvent A and solvent B into the gradient loop. When sample loading is finished, the gradient loop is switched on-

line and the gradient is pushed through the column. After the run is complete, the gradient loop is switched off-line. The sample loop can be switched off-line any time after sample loading is complete.

The gradient loop is filled in the reverse direction from that used to push the gradient through the column. Thus, there is no time delay in the start of the gradient when the loop is brought on-line even when the loop is not completely filled. However, run-to-run reproducibility is improved if the volume of solvent delivered by the gradient program exceeds the capacity of the loop. An extra portion of solvent with the same composition as the front end (part that first enters the column) of the gradient is included to compensate for the small dead volume of the valve and connecting tubing. This portion is termed front end insurance (FEI) and typically has a volume of 5–10 μL. For optimum performance, it is advisable to match the size of the gradient loop to the size of the column used for the separation (see Experimental Methods section and Table 1). The length-to-inner diameter ratio for the gradient loop is a compromise designed to minimize diffusion while at the same time avoiding excessive back pressure during the gradient formation step. Typically, 1 min is required to fill the gradient loop. Even for narrow bore gradient loops, there is always some erosion of the back end of the gradient where it meets with solvent A from the high pressure syringe pump. An extra portion of the final gradient composition [termed back end insurance (BEI), typically 40 μL] is included to protect that portion used to elute the sample. In addition to diffusion, there is some degradation in the gradient profile as the result of mixing when the gradient displaces whatever solvent is already present in the loop. The reverse gradient formed by diffusion at the end of the gradient provides for an easy transition back to initial conditions at the end of the run. However, no changes in column performance have been noted when the gradient loop is switched off-line and 100% solvent A is pumped directly onto the column. It is accepted practice in normal scale HPLC to avoid abrupt changes in solvent composition that result in strong thermal gradients. Such gradients are thought to disturb the column packing and degrade column performance. In capillary columns, heat generated as the solvent front passes through the column rapidly dissipates and damage to the column bed is less likely. It has been our experience that a run can be aborted at any time and initial conditions can be established quickly without any adverse effects.

For protein separations on reverse phase columns, the sample is generally loaded onto the column by using a solvent with a high aqueous content. Except for small, hydrophylic peptides, there is no movement of the sample through the column until the introduction of the gradient. In our system, the sample loop is mounted on a valve upstream from the gradient loop valve. This eliminates the need to push the gradient through this valve. Because only solvent A flows through the sample loop from the high pressure syringe pump, it is important to flush the loop manually to remove any components that might adhere to the surfaces of the sample loop and valve and contaminate the next sample. This is generally done by using several loop volumes of solvent B, followed by 1–2 loop volumes of solvent A, and can be done anytime the loop is off-line and before the next sample is loaded. Peptide and protein samples are often loaded in large volumes of solvent and concentrated on the column. To save time, samples are loaded at a flow rate 5–10 times higher than that used for elution. The volume of the gradient valve with the loop off-line is small in comparison to the volume of sample normally loaded and makes an insignificant contribution to the dead volume of the system. The low pressure syringe pumps are programed to refill before each gradient profile. The high pressure syringe pump has a volume of 100 mL and only needs to be refilled every two or three weeks. This is done manually on our system, although the ISCO pump can be purchased with the option to monitor the volume of the syringe and an automatic refill procedure could be programed into the software. Check valves serve to prevent flow in the wrong direction during either the refill or pumping operation. All other valves except the sample injector are pneumatically actuated. The same helium source used to degas the solvent bottles is used to actuate the valves.

The high pressure syringe pump is operated in constant pressure mode. Most commercial HPLC pumping systems operate in a constant flow mode. Previously [11] we showed that there is no significant difference between constant pressure and constant flow operation when peptides and proteins are separated by capillary HPLC. True constant flow operation on a capillary scale, although theoretically possible, is very difficult to achieve. There is no direct measurement of the actual flow from the column and no feedback to the pump to maintain that flow rate. The piston is simply moved at a constant rate, and it can take a surprisingly long time for a system to come to equilibrium and measured flow rates can change considerably during the course of a run. On the other hand, pressure can be monitored constantly and maintained at a set value by the pump. There is a change in flow rate during the course of the gradient due to the change in solvent viscosity. This change is reproducible and has no adverse effect on the separation. Constant pressure operation is sensitive to flow problems caused by sample precipitation or other blockage in the tubing or on the column. To calculate the volume of the gradient to be delivered by the low pressure syringe pumps the data system assumes the gradient will be pushed through the column at a constant flow rate.

System Performance

The main concern for a system that uses preformed gradients is the change in the gradient profile that

results from storage in the loop. The differences observed between the gradient formed by the syringe pumps and that eluted from the column (Figure 3) are greatest at the sharp boundaries of abrupt solvent changes. The longer the run, the longer the gradient is in the loop and the greater the change. Step gradients, defined as gradients formed by discrete changes in solvent composition as opposed to a linear or exponential ramp between two compositions, are seldom used for actual separations, but are often used to demonstrate the performance of commercial pumping systems. A step gradient is badly deformed during formation and storage in the loop. Mixing in the tee connection and diffusion in the loop tend to smooth out imperfections in the gradient as it is formed and eliminate the need to use a dynamic mixer. The programed gradients illustrated in Figures 2 and 3 are typical of those commonly used on our system, where the separation occurs during the linear ramp portion followed by a portion with high organic content to wash the column.

For the majority of LC/MS analyses, retention time reproducibility is of little consequence. The mass spectrum is a much more powerful means of identification of mixture components than the time of elution. As a consequence, in our lab there is little concern for factors such as column equilibration times between runs, which affect peak retention times. Even for LC tandem mass spectrometry analyses, the instrument is programed to automatically switch between mass spectrometry and tandem mass spectrometry modes based on real time analysis of the spectrum collected in the previous scan [13]. However, for many mass spectrometers, tandem mass spectrometry data for multiple components during a LC separation must be collected as a series of timed events, and in this situation the reproducibility of the separation is important. As variation in retention time increases, the width of the preset window for each tandem mass spectrometry measurement increases and the number of components that can be analyzed during the course of a run decreases. Retention time reproducibility for the gradient loop system is illustrated by the separation of the peptides derived from the enzymatic digestion of cytochrome *c* by Endo Lys C (Figure 4). The mean retention time calculated from six identical runs for the numbered components in the mixture are given in Table 2 along with the range and standard deviation. If we assume any one of the runs was used to set tandem mass spectrometry data collection windows for the remainder of the runs, a 40-s window for precursor ion selection would be sufficient to obtain product ion spectra for all but the first two components in the mixture in every instance. Even the first two components would be missed only occasionally with a 40-s window. For these runs, the column equilibration time was kept constant (15 min between runs). Also, it is important to allow enough time (approximately 15 s) after the syringe pumps fill the gradient loop for the pressure to equilibrate. Otherwise, there is significant variation in the position of the gradient in the loop and a corresponding shift in the absolute peak retention times.

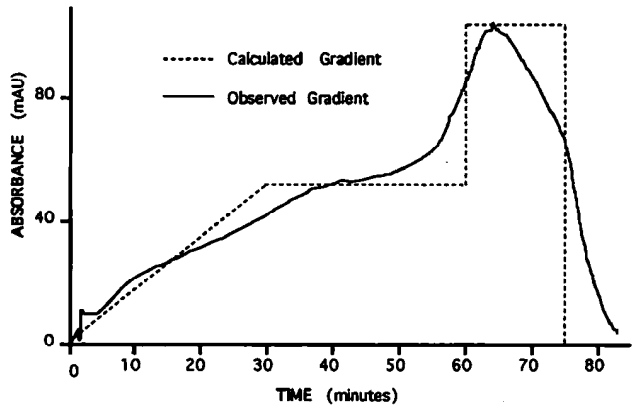

Figure 3. Difference between the calculated and observed gradient profile for a typical gradient loop injection. The UV absorbance was obtained by inclusion of 5% acetone in solvent B and monitoring at 200 nm. Other solvent components are as described in the Experimental Methods section. The discontinuity at the beginning of the gradient is an injection artifact attributed to acetone initially retained on the column, which then elutes as the proportion of solvent B increases. The programed gradient was 2–62% B over 30 min, 30 min of 62% B (BEI), and 20 min of 100% B (column wash). The absorbance was measured at the end of a 15-cm × 0.25-mm packed column with an elution flow rate of 2 μL/min. The gradient loop was 150 μL (0.01-in. internal diameter). UV detector response (*y* axis) is in terms of milli absorbance units (0.001 AU) designated as mAU. The calculated gradient assumes a constant column flow rate. The expected UV absorbance was determined by the isocratic delivery of 62% and 100% of solvent B through the column and detector.

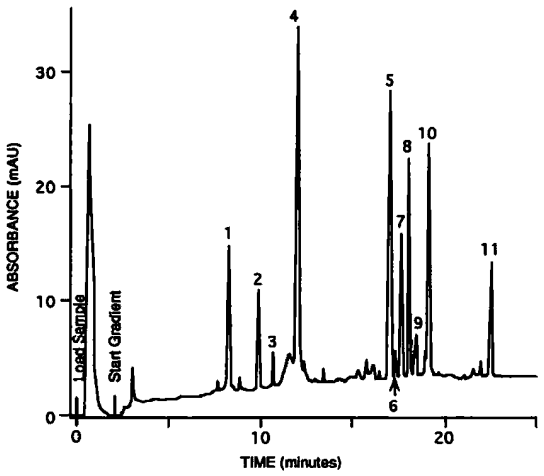

Figure 4. UV detector chromatogram for the HPLC analysis of the peptide mixture from the Endo Lys C digest of cytochrome *c*. A 10-pmol sample of the digest mixture in 1 μL of solvent was loaded at 20 μL/min. Peptides were eluted from the column with a gradient of 2–82% solvent B over 60 min at a flow rate of 2 μL/min. This is one of six runs used to determine retention time variations (Table 1). In this instance, a 15-min delay between runs was used for column equilibration to increase retention time reproducibility.

Table 2. Retention time variation for peaks observed in the chromatographic separation of peptides derived from the Endo Lys C digest of cytochrome *c*[a]

Peak	Retention time (seconds)		
	Mean	Range	Std. dev.
1	376	40	15.6
2	466	25	9.0
3	508	18	8.4
4	580	19	7.8
5	880	19	6.6
6	904	19	7.2
7	925	18	6.6
8	950	20	6.0
9	976	18	7.2
10	1017	18	6.6
11	1215	17	7.2

[a] The mean value is the average for six consecutive runs. The range is the difference between the longest and shortest time. Peak numbers correspond to those in Figure 4. Conditions for the analysis are described in the caption for Figure 4. Retention times are measured from the start of the gradient.

For normal operation with the standard electrospray ion source, 250-μm i.d. columns (2 μL/min flow rate) provide excellent sensitivity and trouble-free operation. With 500-μm i.d. columns (20 μL/min flow rate), it is possible to split the flow from the column and collect fractions that correspond to the LC/MS run to be used for further analysis. The gradient loop system has been used successfully with columns as small as 50-μm i.d. (100-nL/min flow rate). Such work requires the use of narrow diameter tubing to minimize dead volume and a smaller diameter (10–50-μm o.d.) electrospray needles (drawn from FSC tubing). The gain in sensitivity is significant, but needles with these dimensions are very susceptible to plugging.

The advantages of pressure programing to increase the efficiency of LC/MS analyses are illustrated by using the same cytochrome *c* digest mixture (Figure 5). If the sample is loaded at the normal running pressure (400 lb/in.^{2}) for good chromatography, a total of 45 min is needed to complete the analysis (Figure 5c). If the sample is loaded at 10 times the normal flow rate, the analysis time is reduced to 32 min (Figure 5b). The time savings is dependent on the volume of sample to be loaded. In this case, only 1 μL of sample was used and the loading time is the time needed for the sample solvent plug to arrive at the detector. This provides a convenient marker for determination of when the loading operation is complete. The analysis time can be shortened to 25 min by starting the gradient while the pressure is being ramped from the loading pressure to the running pressure (Figure 5a). By cutting analysis times by 40–50%, the productivity of the mass spectrometer is increased greatly with no loss of chromatographic resolution.

By using the same technique, small quantities of peptide and protein that require little or no chromatographic separation can be analyzed quickly. Three successive analyses of cytochrome *c* can be done in less than 25 min (Figure 6). The quality of the mass spectra obtained by averaging scans over the peak is excellent (Figure 7). This technique has several advantages over simple flow injection analysis. (1) Peptide and protein sample components are separated from components such as salts that often compromise the quality of the analysis. (2) Low sample volumes can be injected without loss of sensitivity due to diffusion. (3) Larger volumes of dilute sample can be loaded and eluted at a higher concentration. (4) Gradient elution generally eliminates sample carryover that can occur when proteins adhere to tubing surfaces. (5) Finally, the same instrument configuration can be used to do long chromatographic separations of complex mixtures and rapid analyses of previously isolated compounds.

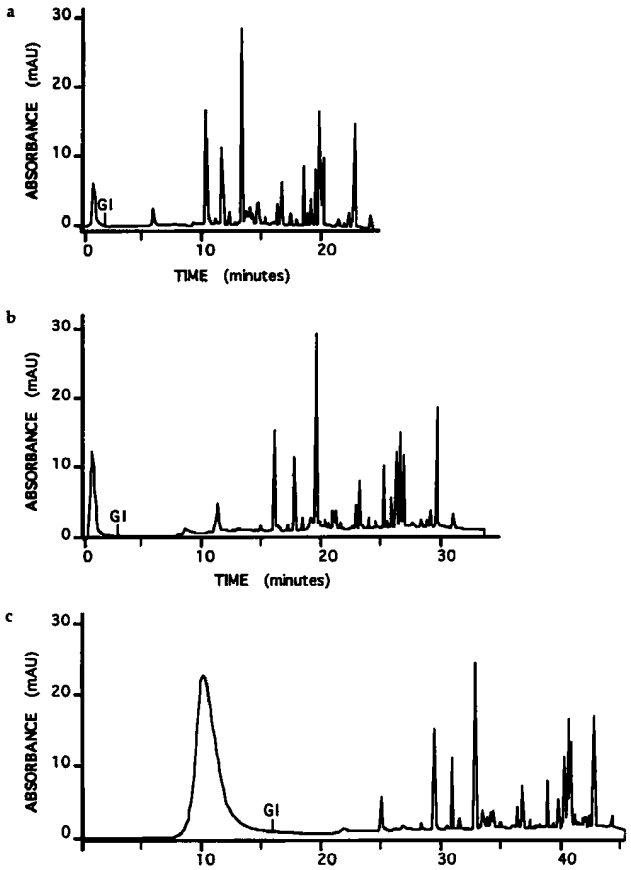

Figure 5. UV detector chromatogram from the LC/MS analyses of Endo Lys C digest of cytochrome *c* with different pressure programs. GI marks the point the gradient is injected. (a) The sample was loaded at 4000 lb/in.^{2} and gradient elution started at the beginning of the pressure ramp from 4000 to 400 lb/in.^{2}. (b) The sample was loaded at 4000 lb/in.^{2} (20 μL/min) and gradient elution started after the pressure ramp from 4000 to 400 lb/in.^{2}. (c) The sample was loaded and eluted at the normal running pressure of 400 lb/in.^{2} (2 μL/min).

Conclusion

The gradient loop HPLC system described in this work provides a convenient, low cost, and effective means to

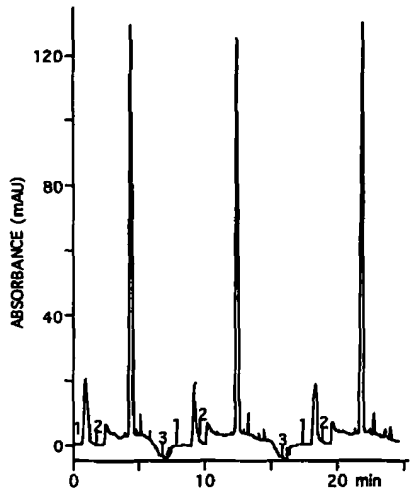

Figure 6. UV detector chromatograms from the repeated LC/MS analyses of equine cytochrome *c*. The numbers in the figure refer to the following events: (1) sample solution (5 pmol in 1 μL) loaded at a flow rate of 20 μL/min (4000 lb/in.^{2}); (2) switch in the gradient loop at the beginning of the 1-min pressure ramp from 20 to 2 μL/min with subsequent elution of sample at 2 μL/min by using a gradient of 5–75% solvent B over 15 min; (3) switch gradient loop off-line and return to initial conditions at 20 μL/min.

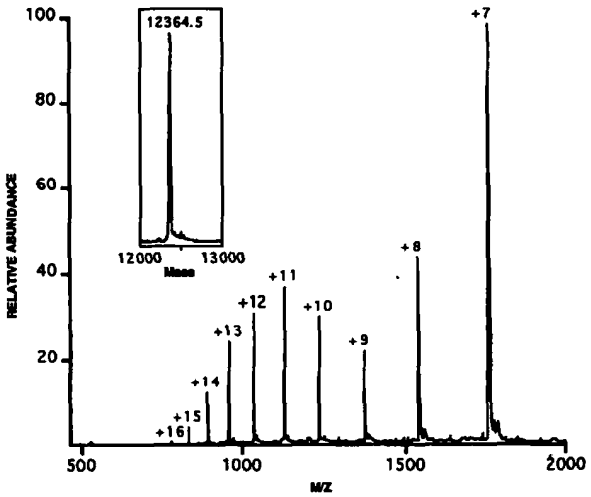

Figure 7. Positive ion electrospray mass spectrum of cytochrome *c* from the LC/MS analysis shown in Figure 6. The spectrum is the average of scans over one chromatographic peak. The insert is the conversion of the normal mass-to-charge ratio scale to molecular mass.

couple capillary liquid chromatography to mass spectrometry. The absence of split flows simplifies operation and greatly reduces solvent consumption. The use of pressure programing to control flow rates dramatically increases instrument productivity by reducing the sample loading times and the delay between the start of the gradient and its arrival at the column. Elution time reproducibility is sufficient to program multiple tandem mass spectrometry analyses as timed events during the course of a single LC/MS run.

Acknowledgments

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