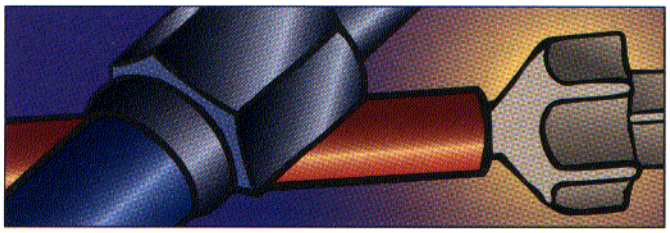
LC Troubleshooting



When Good Isn't Good Enough

Daniel H. Marchand, Peng-Ling Zhu, and John W. Dolan

For some applications, instrument manufacturers' specifications provide inadequate performance.

In our laboratory, we analyze many protein and peptide samples by reversed-phase liquid chromatography (LC). In most ways, these samples exhibit the standard reversed-phase behavior most of us have come to expect. For example, increasing the organic solvent content (percentage of solvent B) of the mobile phase reduces retention. However, in other ways, these samples have very different behavior from the small molecules most of us encounter on a daily basis. This behavior is common for proteins and peptides; it occurs in any larger molecular weight sample, as we will see later.

THE BACKGROUND

Our sample is a synthetic peptide with a mass of approximately 1000 Da. We developed a method for analyzing this compound that comprised a very shallow gradient, typically 19–24% acetonitrile–0.1% trifluoroacetic acid in water over 30 min at a flow rate of 1.5 mL/min. We used a 25 cm × 4.6 mm, 5-μm d_p C18 column at a temperature of 35 °C. Detection was performed at 215 nm. We used a two-pump, high-pressure mixing LC system to generate the gradient. We had validated this method, and it worked well for several months in routine use. Our system-suitability specification called for retention-time reproducibility of better than ±0.25 min for three consecutive standard injections.

THE PROBLEM

The method worked very well for an ongoing stability study. The LC system was used for other assays between the stability study's 30- and 60-day sample points. When the time came for the analysis of the 60-day samples, we installed the column that we had been using for the method and prepared a batch of mobile phase. After running the system-suitability standards, we saw very poor retention reproducibility. The retention times seemed to drift, as illustrated for the expanded portion of three runs overlaid in Figure 1a. These three peaks represent the third through fifth consecutive injections of the standard. The retention time increased with each injection. A retention variation of more than 2 min clearly was unacceptable, so we set out in search of the root of the problem.

THE SEARCH

When retention times drift from run to run with gradient methods, one of the first variables to suspect is poor column equilibration between runs. We checked this variable by extending the equilibration time but observed no improvement. Occasionally some samples require that you dope the column with enough sample to block specific active sites. We hadn't encountered this problem during the development or use of the assay, but it was worth exploring, so we made several large sample injections. But this expedient did not improve the situation.

Chromatographers often blame their columns for nearly any problem they encounter in LC systems. We didn't suspect that the column was bad because the standard measures of column performance — peak width, tailing, and back pressure — were normal. However, exchanging the column was easy, so we installed a new column. We observed the normal small retention changes expected with a column change, and the retention variability remained. We reinstalled the original column.

By this time, we had injected enough samples to realize that the retention shift was not progressive, but random — we had been misled by the first few injections in concluding that retention drifted in one direction only. This observation led us to suspect the LC system hardware. The easiest way to eliminate a hardware problem is to move the column to another LC system and observe the results. When we did this, we saw a little improvement but not sufficient improvement to pass the system-suitability test, so we returned to the original system.

HARDWARE CHANGES

With an on-line mixing LC system, changes in retention can be caused by flow-rate changes, mobile-phase blending problems, or a combination of both. When using high-pressure mixing, as with our LC system, both causes can be traced to flow-rate problems. We checked the flow rate of each pump by setting the flow to 1.0 mL/min and timing the collection of 10 mL of mobile phase in a volumetric flask. Both pumps passed the test within the manufacturer's ±2% accuracy specification. However, a problem with a timed-collection test — such as the one we used — is that it integrates the flow rate over a long time — 10 min in our case. This long time period can hide small changes in flow rate that are averaged out over time.

Another way to make a rough estimate of the flow rate is to look at the peak at the column dead time, t_0. This peak is unretained, so it should be uninfluenced by the mobile-phase composition. After taking a close look at the t_0 peaks for the chromatograms in Figure 1a, we obtained the results of Figure 2a. The peaks had a retention range that just exceeded the manufacturer's specifications of ±0.3% precision for the pump flow rate. Considering that our measurement technique also compensated for any timing errors from the autosampler, the performance may have been okay.

At this point, the pump performance seemed to be marginal at best. Because we were under time pressure to get the sample results to our client, we abandoned the *Change One Thing at a Time* rule and refurbished the liquid ends of both pumps in one step. Our service included replacing all eight check valves (two outlet and two inlet valves on each pump) and four pump seals.

A PARTIAL VICTORY

We anxiously reinjected our system-suitability standards, hoping that the problems would have disappeared. The improvement we saw was significant, but the method was still out of specifications. The results of Figure 1b show that the retention time variation had dropped in half but still was greater than ±0.5 min. Simi-

John W. Dolan
LC Troubleshooting Editor

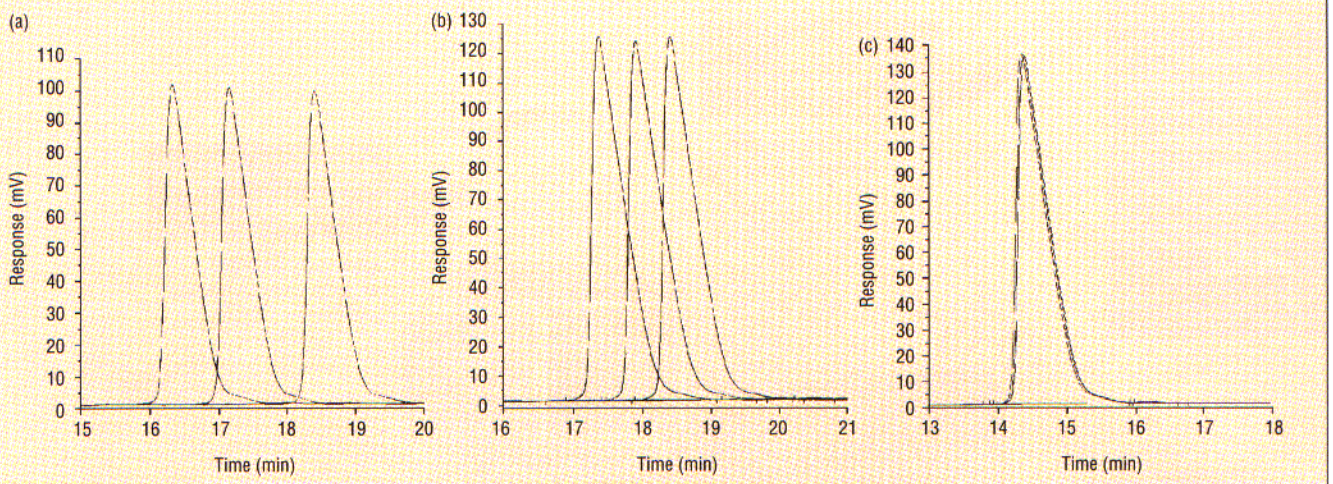


FIGURE 1: Expanded chromatograms from sets of three consecutive injections of peptide standard. Shown are chromatograms generated (a) using the original LC configuration (2.1-min retention range), (b) after replacing all check valves and pump seals (1.0-min retention range), (c) after replacing all check valves and pump seals and using premixed mobile phase (retention range less than 0.1 min). See text for details.

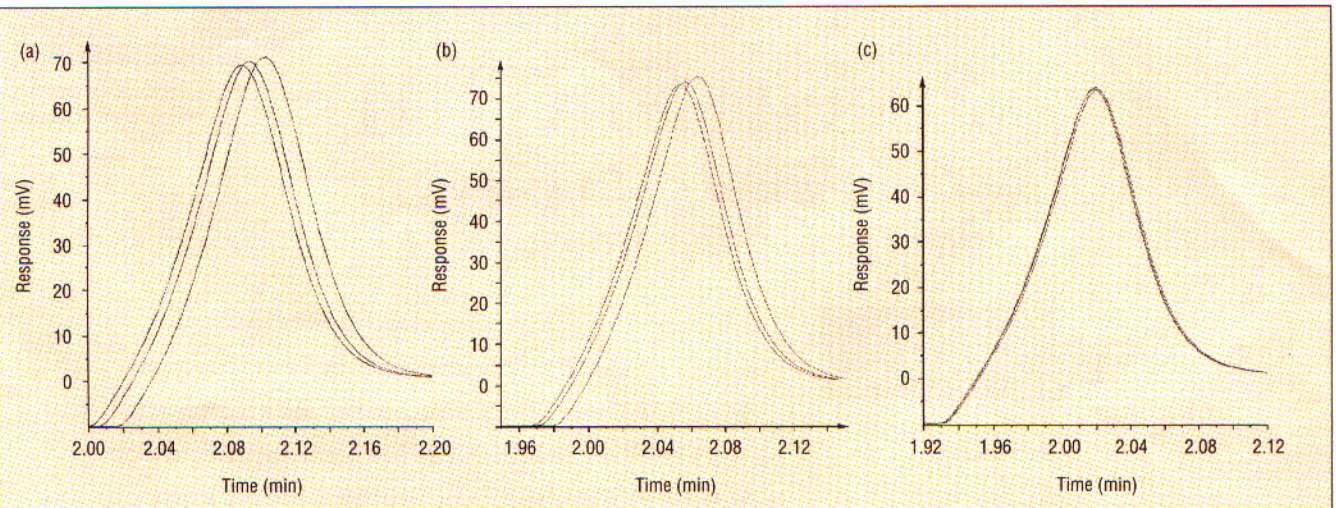


FIGURE 2: Expanded chromatograms showing the t_0 region of three consecutive injections of peptide standard. Shown are chromatograms generated (a) using the original LC conditions (0.014-min retention range), (b) after replacing check valves and pump seals (0.010-min retention range), (c) after replacing all check valves and pump seals and using premixed mobile phase (retention range less than 0.001 min). Data were taken from the chromatograms shown in Figure 1. See text for details.

larly, the variation in retention of the t_0 peak had dropped to well within the manufacturer's specification (Figure 2b).

At this point, it appeared that the LC system was performing to within the manufacturer's specifications. A recent system check for gradient linearity and accuracy had passed the manufacturer's specifications for these parameters. Now we were stuck with a method that previously worked on this system, and the system was performing according to its design specifications by all our measurements, but it still was not good enough for our needs.

AN ADDITIONAL PLOY

We knew the method was very sensitive to small changes in the gradient slope, so we suspected that this sensitivity was the source of the remaining variation. We couldn't improve the system performance directly, so we took an indirect approach. The gradient we were using covered a range of 5% solvent B in 30 min or approximately 0.17%/min. This gradient was equivalent to changing the flow rate by 5% during 30 min, which certainly must challenge the mechanical design of the pump.

A simple way to overcome this problem is to premix the mobile-phase components. So rather than using the original solvent A of 0.1% trifluoroacetic acid and solvent B of 100% acetonitrile, we blended the solvents to have solvent A comprise premixed 10:90 acetonitrile–0.1% trifluoroacetic acid and solvent B comprise 30:70 acetonitrile–0.1% trifluoroacetic acid. We reset the controller range to 40–65% solvent B in 30 min, a nominal change of 25% during 30 min. This gradient was much less demanding for the LC hardware.

This change theoretically should produce a fivefold improvement in performance; in fact, the improvement was much greater. Figure 1c shows that the three replicates almost are on top of each other, whereas the difference in the t_0 peaks shown in Figure 2c is unmeasurable. (The reduction in t_0 variability probably was caused by small but sig-

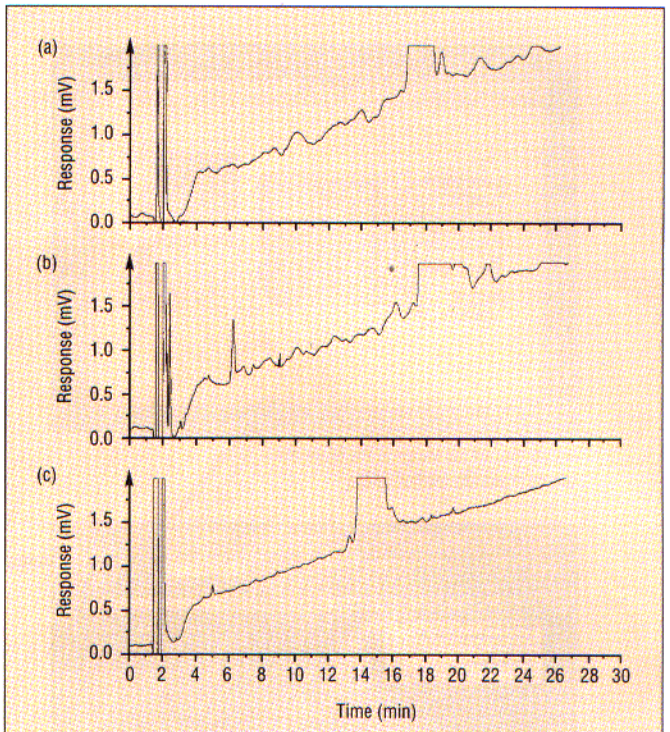


FIGURE 3: Expanded baselines of three chromatograms with conditions corresponding to (a) Figures 1a and 2a, (b) Figures 1b and 2b, and (c) Figures 1c and 2c. See text for details.

nificant retention of the peak, which makes t_0 sensitive to minor mobile-phase changes.) Our problem was solved, and we were able to complete the required analyses with ease.

Figure 3 demonstrates an additional benefit derived from premixing the mobile phase. It shows greatly expanded baselines obtained with the three sets of conditions used to generate the chromatograms shown in the other figures. We can see that the repair of the pump in Figure 3b resulted in some improvement in the frequency of the baseline fluctuations but little change in the magnitude of the baseline noise when compared with the original conditions of Figure 3a. After we premixed the mobile phases, we observed the baseline of Figure 3c, which shows a significant improvement in the magnitude of the baseline noise.

This result suggests that the frequency of the noise is somewhat dependent on the condition of the pump hardware but that the magnitude of the noise is a mixing phenomenon. One way to reduce mixing problems is to premix the mobile phase as we did here. An alternative is to use a larger mixer, an option that we did not explore.

GRADIENT SENSITIVITY

At the beginning of this column, we alluded to the increased retention-time sensitivity for proteins and peptides compared with small molecules under similar gradient conditions. Figures 1b and 1c illustrate this observation. If we ignore the variation in retention times in Figure 1b, we see that the average retention time is 17.9 min.

In the chromatogram of Figure 1c, we changed the gradient program from 18–23% solvent B in 30 min to 19–24% solvent B in 30 min. This small change would have minimal influence on the retention of most small molecules, but it caused a shift in retention of approximately 3.5 min (20%) for our peptide. This behavior is common, and we often see an order-of-magnitude increase in sensitivity to mobile-phase changes with larger molecules such as our peptide. For this reason, it is prudent to take extra care in controlling all the chromatographic parameters.

CONCLUSIONS

Certain sample types place increased demands on the performance of LC systems, and in some cases, a system that performs according to the manufacturer's specifications is inadequate for required separations. Techniques such as premixing mobile phases to improve mobile-phase blending precision are very practical solutions to vexing problems.

In closing, we should point out that even at its worst, this particular LC system performed routine small-molecule assays with a high degree of precision. Only the peptide sample pushed the system performance to its limits.

Daniel Marchand is the technical director for laboratory services at LC Resources Inc., McMinnville, Oregon. Peng-Ling Zhu is a professor of chemistry at Lanzhou University, Lanzhou, China; a visiting scientist at Linfield College, McMinnville, Oregon; and a visiting fellow at LC Resources Inc., McMinnville, Oregon.

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