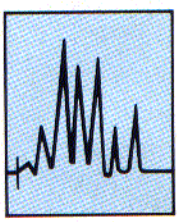
L C T R O U B L E S H O O T I N G

Mobile Phase Proportioning Problems: A Case Study

JOHN W. DOLAN



This month's installment of "LC Troubleshooting" discusses a problem that we recently encountered in our laboratory while analyzing peptide samples by liquid chromatography (LC). Because we have worked primarily with low–molecular-weight samples in the past, this problem caught us by suprise. Workers who commonly analyze peptides, proteins, and other medium– to high–molecular-weight samples may already incorporate the practices recommended here, or they may be fighting the same problems without a successful solution.

THE SYSTEM AND THE SAMPLE

We had just begun a project that involved analyzing a sample of synthetic peptides by reversed-phase LC. The system hardware was about four months old and had been used extensively without problems. The system consisted of a two-pump binary-gradient liquid chromatograph with rapid-refill pump heads and a mechanically stirred mixer. The manual injector and column were maintained at 30 °C in a column compartment. A variable-wavelength UV detector was set to 210 nm, and the chromatographic output was recorded by a computerized data system. A back-pressure regulator set to ~50 psi was used after the detector to minimize bubble problems.

The mobile phase reservoirs were filled with HPLC-grade water (the A solvent) and HPLC-grade acetonitrile (ACN, the B solvent), each containing 0.1% trifluoroacetic acid (TFA). Because high-pressure mixing was being used, the mobile phases were not degassed. The method was similar to those commonly used for peptide analysis: a shallow gradient from 5% to 80% B over 75 min (1%/min).

INITIAL BASELINE PROBLEMS

From the outset, we encountered problems with the baseline. The sample baseline shown in Figure 1a shows a typical "bad" case of baseline noise with an amplitude of ~1.2 milliabsorbance units (mAU) at 210 nm. We observed that the frequency of the sinusoidal cycle roughly corresponded to the cycle time of the A-pump piston, which led us to suspect a check-valve problem with the A pump. Replacing the inlet and outlet check valves in both pumps resulted in no performance improvement. After reinstalling the original check valves, we decided to check for inadequate mixing. Adding a 2-mL static mixer or a packed-bed mixer did not improve the situation.

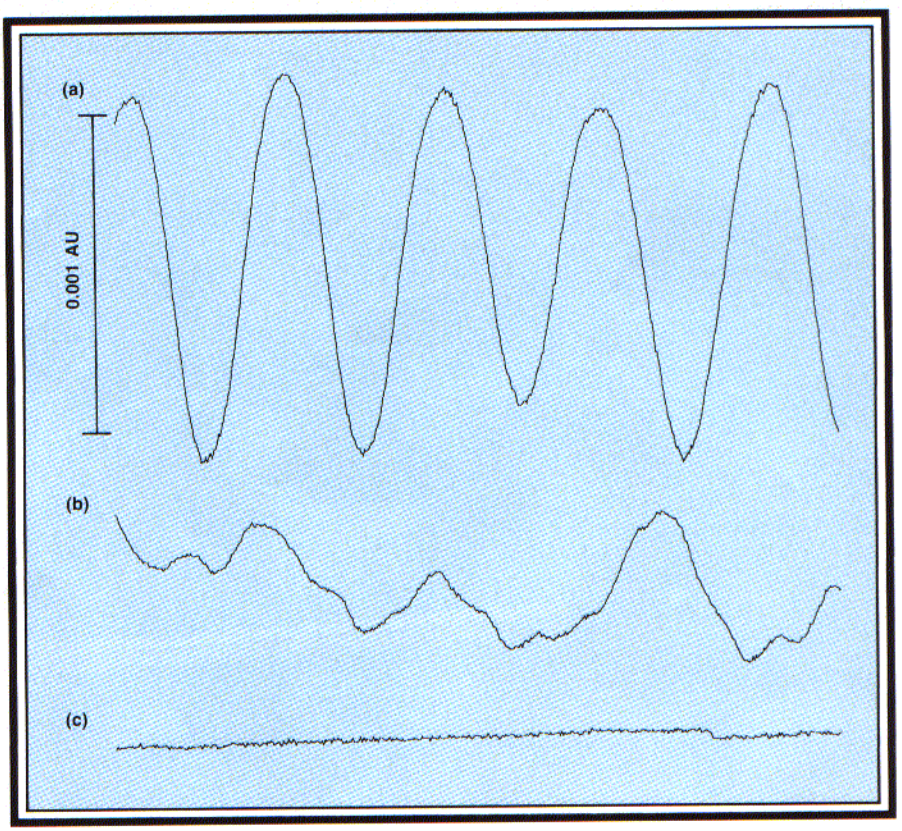


FIGURE 1: (a) Typical baseline noise (~1.2 mAU peak-to-peak) observed at the onset of the problem, measured at 210 nm; (b) noise improvements (~0.4 mAU) resulting from premixing the mobile phase, matching A- and B-solvent absorbances, and changing to 220 nm for detection; (c) baseline (~0.02 mAU) with degassed mobile phase.

At this point, we had run out of ideas, so we contacted colleagues with expertise in instrument design and peptide applications. As one person pointed out, "mixing TFA/ACN mobile phases is probably the most difficult test of gradient mixing systems." We incorporated three of our colleagues' suggestions, and the baseline improved (Figure 1b). First, we made the absorbances of the A and B solvents more equal by using 0.115% TFA in the water and 0.1% TFA in the ACN. This reduced the amplitude of the noise because there was less difference in absorbance between the two solvents. Second, we premixed the solvents in order to reduce initial immiscibility problems that may occur when pure water and pure ACN are mixed. Thus, the A solvent was premixed to 5:95 ACN/water and the B solvent to 80:20 ACN/water. Finally, we increased the operating wavelength from 210 nm to 220 nm, which further reduced the noise problem. While these changes did not completely eliminate the problems we encountered, they did reduce the baseline noise by a factor of three (from 1.2 mAU to 0.4 mAU; compare Figure 1a with Figure 1b).

RETENTION REPRODUCIBILITY PROBLEMS

With the changes mentioned above, we were able to successfully run a number of samples in preliminary retention scouting studies. However, when it came time to check retention-time reproducibility, the results were unsatisfactory. The first two runs of Table I show the magnitude of the problem. In each set of three runs, one or more of the bands show acceptable reproducibility (retention-time range of <0.1 min), but one or more bands show very bad reproducibility (>0.5 min). Furthermore, the bad bands change between the two sets of runs. At this point, we tried several different mobile phase additives to try to reduce unwanted interactions between the sample and the column, but this did not improve the reproducibility. Next, we tried a different column packing type, which also had no effect. Finally, we tested the reproducibility using small-molecule test probes (toluene, acetophenone, etc.) with the results seen on line 3 of Table I, which plainly show that the reproducibility is satisfactory for small molecules.

TABLE I: RETENTION-TIME REPRODUCIBILITY*

Run	Sample	Retention-time range (min) for band: 1	2	3	4	5	6
1	Six peptide polymers**	0.00	0.58	0.39	0.56	0.35	0.29
2	Six peptide polymers**	0.01	0.09	0.18	1.01	0.61	0.43
3	Six low–molecular-weight test probes†	0.02	0.02	0.05	0.08	0.10	0.07
4	Same as 1 and 2, after mobile phase degassing	0.02	0.02	0.05	0.05	0.04	0.05

* manual injection used in all cases
** duplicate sets of three runs
† range for three consecutive runs

CHECKING FOR PROPORTIONING PROBLEMS

These findings once again made us suspect solvent proportioning problems. We decided it was time to start with the basics and rerun a step gradient between methanol (A solvent) and acetone-spiked methanol (B solvent), as was discussed in an earlier column (1). The results for one of these runs are shown in Figure 2. The step size was fairly even, indicating that the accuracy of the proportioning was not too bad, but the noise on some of the plateaus was not acceptable. At several points (see arrows in Figure 2), the trace looked almost as if there were a bubble in the system (one would expect a bubble to produce a sharper baseline dip). To eliminate any bubble problems, we degassed the mobile phase with vigorous helium sparging.

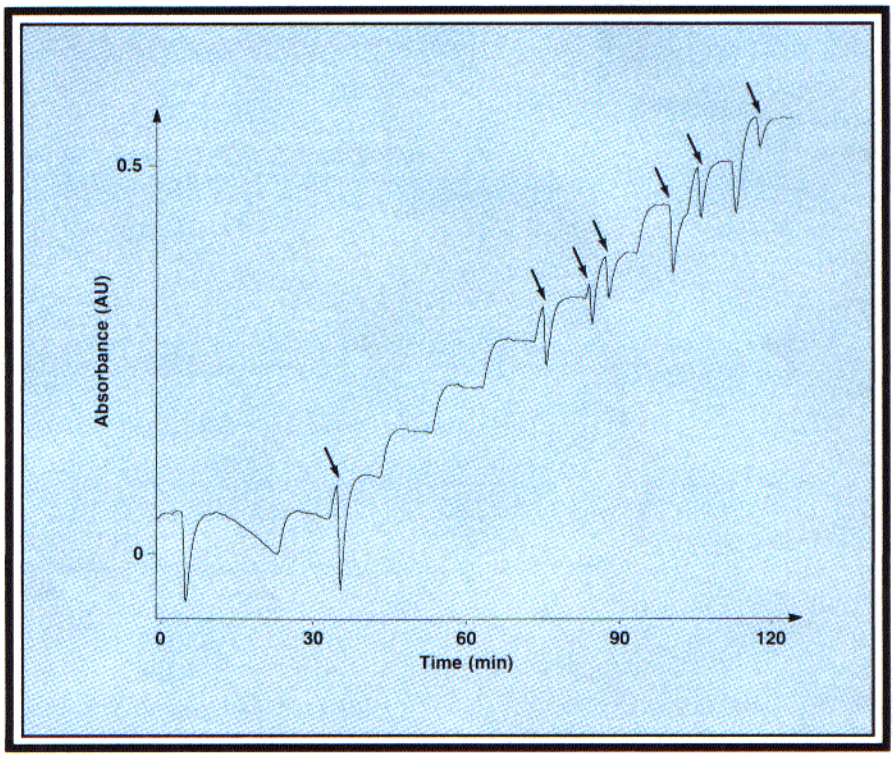


FIGURE 2: Sample step gradient, 30%–40% methanol spiked with acetone. Arrows show areas suggesting bubble problems.

DEGASSING (ALMOST) SOLVES THE PROBLEM

Degassing immediately eliminated the baseline deviations on each plateau, indicating that we were finally onto a solution to our problem. We switched back to the TFA/ACN mobile phase (with premixing and absorbance matching, and run at 220 nm) and reran our peptide standards with degassed mobile phase. The results seen for run 4 of Table I show that we seem to have solved the retention reproducibility problem. Notice that the variation in retention time has improved by an order of magnitude over the earlier runs (compare run 4 with run 2). Furthermore, the baseline noise was reduced by an order of magnitude (to <0.1 mAU) over some of the earlier conditions (compare Figures 1a and 1b with 1c).

At this point, we were able to return to our earlier goal of separating the synthetic peptide mixture. We were using computer simulation techniques (2) to predict retention based on two scouting gradient runs. In general, the predictions of retention times compared quite favorably with experimental runs (for example, ±1% in retention). There were, however, occasional exceptions to the accuracy of the predictions. A sample chromatogram is shown in Figure 3a for a mixture of 23 synthetic peptides. Examining a number of other chromatograms showed that poor retention prediction correlated with a negative dip in the baseline just ahead of the problem bands (noted with arrows in Figure 3). Furthermore, in each case the errors were always in the same direction. That is, the experimental retention times for the problem bands were always longer than the predicted times.

A SOLUTION

From our earlier work with matching the absorbance of the starting A and B solvents, we knew that the B solvent had a slightly higher absorbance than the A solvent. This led us to suspect that once in a while there was a local starvation for the B solvent. Occasional solvent delivery problems often are related to check-valve problems, so we replaced the inlet check valve on the B pump, and the baseline dips disappeared in subsequent runs (see Figure 3b).

A DIAGNOSIS

So what caused all these problems? Our current hypothesis is that the refill cycle of the pump was causing cavitation (solvent outgassing). This, in turn, resulted in the uptake of less than the specified volume of solvent. The delivery stroke repressurized the solvent, forcing any gas bubbles back into solution, but the volume of solvent delivered was less than it should have been. Thus, regular or occasional proportioning errors occurred that were too large to be averaged out in the mixing system. When the starting solvents were

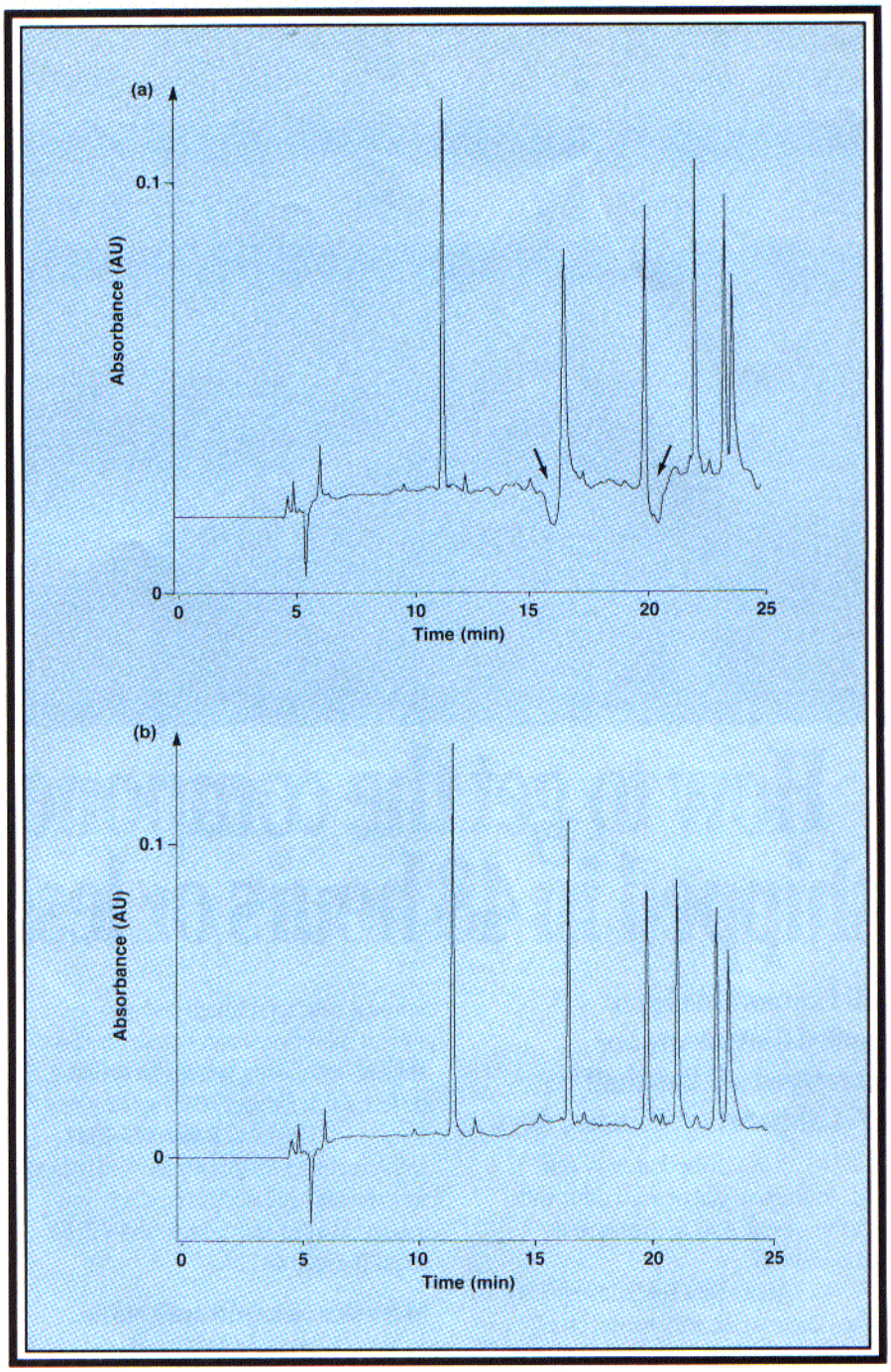


FIGURE 3: Chromatogram of synthetic peptide mixture, 5%–80% ACN (0.1% TFA)/water in 75 min at 1 mL/min. (a) Before check-valve replacement. Arrows note baseline disturbances suggesting acetonitrile starvation just before poorly predicted peaks elute. (b) After check-valve replacement.

thoroughly degassed, no cavitation could occur (the volume of dissolved air in the solvents was drastically reduced), so more accurate proportioning resulted.

Why isn't this kind of proportioning problem commonly encountered with high-pressure mixing systems when degassing is not used? The main reason is that the low–molecular-weight solutes (for example, toluene) typically used to test retention are not very sensitive to small local changes in solvent composition. This is illustrated by comparing the reproducibility data for small molecules (run 3 in Table I) with data for large molecules (run 2). We know from the discussion of solute *S*-values in a previous column (3) that the higher the molecular weight for a solute, the more sensitive it is to small changes in mobile phase composition. In other words, the proportioning problem was there all along, but we never noticed it because the samples that we were analyzing were not affected by mobile phase variations of this magnitude. An extreme example of this sensitivity, in

which proportioning problems made it appear that a single peak was being fractionated into component peaks, was discussed in an earlier column (4).

The presence of a bad check valve at the same time as a degassing problem demonstrates that we can't always count on the basic troubleshooting assumption that only a single source is responsible for each problem. Fortunately, in the present case, the presence of two simultaneous problems did not cause much additional confusion.

A WORD TO THE WISE

What can we learn from all this? First, degassing may be necessary with high-pressure mixing systems, even when the traditional problems of bubbles in the detector are not seen. Certainly, degassing with such systems is strongly recommended when high–molecular-weight samples are to be analyzed.

Vigorous helium sparging of premixed mobile phases or mobile phases containing additives can result in changes in mobile phase composition over a day's time. This may or may not cause retention drift, depending on the sample and mobile phase. One easy way to avoid problems due to excessive sparging is to use a degassing system that maintains a positive head pressure on the helium bottles. Most such systems are designed so that the solvent undergoes initial vigorous sparging, then a valve is closed so that a slight pressure is maintained in the reservoir; as solvent is pumped out, helium enters the headspace to replace the lost volume. Because the total volume of helium sparged through the solvent is low, there is little chance for loss of volatile mobile phase components. Commercially available systems use a pressure-relief valve to prevent overpressurizing the reservoir; homemade degassing systems should be designed with similar precautions.

An alternative solution is to presaturate the helium with the mobile phase. This is done by using two reservoirs for each mobile phase. Sparge the first reservoir in the normal manner; the helium exiting this reservoir is now saturated with the mobile phase. Connect the vent of the first reservoir to the sparging line of the second reservoir, so the second is sparged with this presaturated helium. The solvent from the second reservoir is pumped to the LC system; the composition will not change with continued sparging because no net change in solvent composition occurs.

A final thing to note in this case is the power of predictive software to help diagnose chromatographic problems. Such computer programs are currently available from a few vendors and are under development by most of the major instrument manufacturers. In the future, when most labs will have such software installed, users will be able to quickly compare their results with those predicted by the computer. In cases such as the present example, in which the predicted and experimental results do not agree, problem solving should be simplified.

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(3) J.W. Dolan, *LC•GC* **4,** 1178–1182 (1986).
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"LC Troubleshooting" editor John W. Dolan is president of LC Resources Inc. of Lafayette, California, USA, and is a member of the Editorial Advisory Board of LC•GC.