LC Troubleshooting

Case Study — Irreproducible Retention Times

John W. Dolan

Sometimes more than one solution will correct the symptoms of a problem.

This month's "LC Troubleshooting" column looks at the problem of irreproducible retention times. The column is based on a conversation with a reader about retention-time problems. I gave her some suggestions after she sent an initial letter, and she followed up with a discussion of her attempts. The solution for this problem reminds us that regular preventive maintenance can pay big dividends in reduced downtime.

A READER'S COMPLAINT: SHIFTING RETENTION TIMES

User: I have been using a routine liquid chromatography (LC) method to identify four peaks in a quality-control analysis of a pharmaceutical product. I had used this U.S. Pharmacopoeial (USP) method for many months with no problems, then I noticed that the method was no longer working properly. I observed a twofold retention problem. The most noticeable problem was that the retention time for the last peak shifted from approximately 16.5 min to approximately 9 min. Also, the retention times became irreproducible, with observable peak shifts of as much as 30 s between runs (as illustrated in Figure 1).

The LC system comprises a single pump with low-pressure mixing, an autosampler, a column, and a diode-array detector. I use a 25-cm C8 column with a 2.0-mL/min flow rate for the separation. The mobile-phase reservoirs contain an A solvent of 1.5% diethylamine in water at pH 7.5 and a B solvent of methanol. I use a mobile-phase gradient that begins with a 12-min isocratic hold at 62% B followed by a 2%/min increase to 92% B over the next 15 min. After a final hold for 5 min at 92% B, a gradient returns the mobile phase to the starting conditions. With the method's current problem, all the analytes are eluted during the isocratic hold, whereas the last two analytes should be eluted during the gradient portion of the run.

When I discovered the problem, I started troubleshooting by checking for changes in various operating parameters. First, I verified that all the system settings were correct. I suspected a problem with the mobile phase, so next I carefully prepared fresh A and B components of the mobile phase. This fresh mobile phase failed to improve the variation. Following this test, I replaced the column with a new one. Again, I observed no improvement. I even measured the flow rate, but it was 2.0 mL/min, just as it should have been. With the obvious problem variables examined, I began to look at other parts of the system that could affect retention. I knew that column temperature could affect retention, but I didn't think it could change retention by a factor of two. Even so, I added a thermostated column oven to the system, but it provided no relief. I've run out of ideas; can you suggest how to fix the problem?

John W. Dolan: It looks like you've checked the most obvious sources of error. The way to approach this problem is to examine the factors that affect retention time in a gradient separation. These factors are flow rate, temperature, mobile-phase composition, gradient conditions, and the column. You already have addressed the flow rate, the column temperature, and the column. It appears that you made no mistakes making up the mobile-phase components, because fresh mobile phase did not change the results. You also checked the settings, so we can assume they are correct.

The only thing remaining is a possible change in the mobile phase entering the column. That is, although the settings are correct, the correct mobile-phase composition is not reaching the column. We have two indications that something is wrong with the mobile phase. First, for a twofold change in retention to occur, the mobile phase's organic composition would have to change by more than 5%. Second, the retention fluctuations you observed suggest a mobile-phase proportioning problem.

The wrong mobile-phase composition can result from one of two possible failures (assuming that the reservoir contents were prepared correctly). Both of these failures stem from problems with the low-pressure mixer. If a restriction occurs in one or both solvent lines leading from the reservoir to the mixer, the proportion of each solvent may be wrong. Alternatively, a malfunction in the controlling software or a mechanical problem with the mixer could yield the same results. Next, we'll look at two general problem areas: mobile-phase starvation and mixer malfunction.

Checking the reservoir lines: The supply of one or both mobile-phase components could be limited by a blocked or partially blocked inlet-line frit in the reservoir, by a blockage or crimp in the tubing leading from the reservoir to the mixer, or by improper reservoir venting (or pressurizing if you are using a sealed system).

You can check for adequate solvent flow by removing the fitting that connects the inlet line with the mixer. The solvent should siphon freely — if it does not, you have a restriction. Loosen the reservoir cap if it is sealed (this will relieve any vacuum in the reservoir if venting or insufficient pressurization is a problem), and the solvent should siphon freely. If flow is still restricted, remove the inlet-line frit, prime the line, and check for siphoning again. If the line siphons, the frit is blocked; if the flow is still restricted, replace the tubing. If

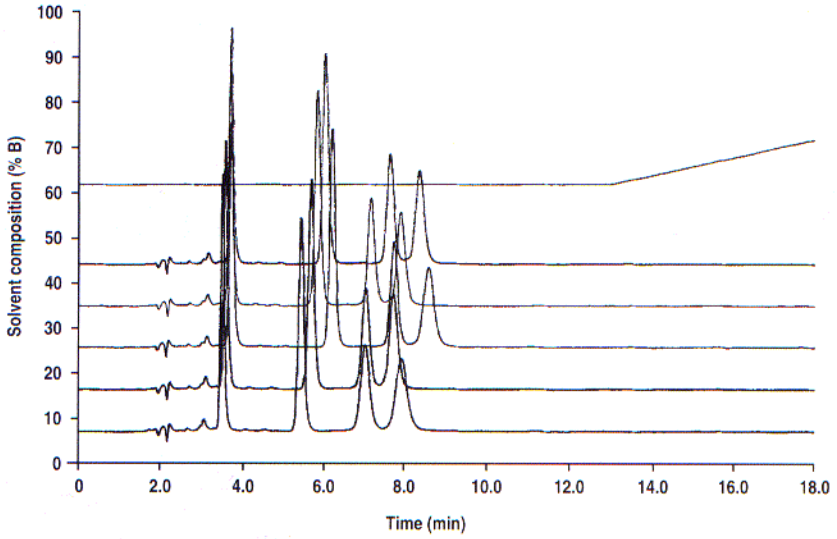

FIGURE 1: System-suitability check for four consecutive injections of a four-component standard mixture. Conditions are described in the text.

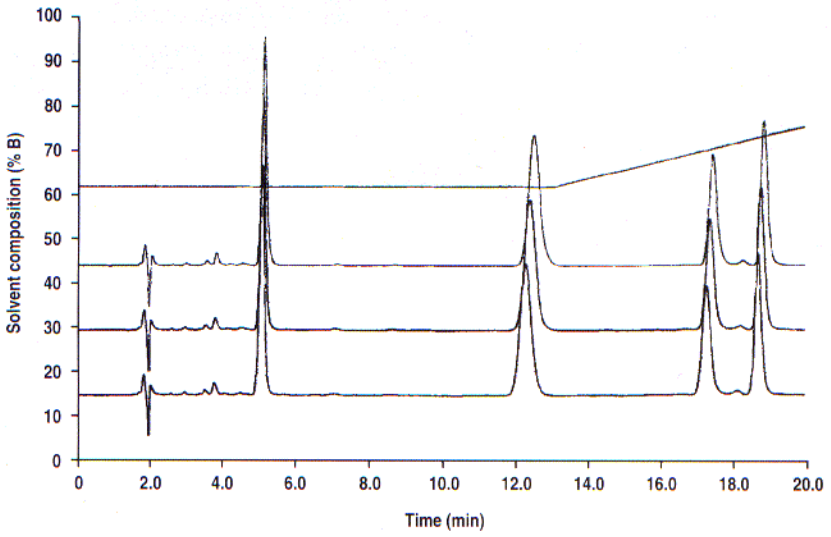

FIGURE 2: Three consecutive runs under same conditions as Figure 1, except for removal of the frit from the A solvent inlet line.

you replace the inlet-line frit, use a 10-μm porosity frit. Smaller porosity frits are available, but they will become blocked more quickly, and they offer no advantages for trapping the kind of debris that gets into the reservoir.

Try premixing: If you verify a sufficient solvent supply and the problem persists, you can improve the proportioning accuracy by premixing the mobile-phase components in each reservoir. For example, you could premix 62% methanol for the A reservoir and 92% methanol for the B reservoir (with aqueous diethylamine as the balance in each reservoir). With these premixed mobile phases, you can adjust the controller to run the gradient from 0–100% of the B reservoir with the appropriate adjustments at the controller — an isocratic hold for 12 min at 0% B followed by a 0–100% B gradient over 15 min. By premixing the mobile phase, you should reduce mechanical proportioning errors by approximately 70%. In the original method, a 1% change on the controller made a 1% change in the percentage of the organic mobile phase ([100% final − 0% initial] × 1% change). Now, the same 1% change will change the organic percentage by only 0.3% ([92% final − 62% initial] × 1%).

User: I tried two experiments. First, I removed the inlet frit in the A reservoir. The retention times are normal, although I observed a little retention-time variation (Figure 2). Could this variation be caused by a partially blocked frit in the B reservoir? Next, I premixed the A and B solvents as you suggested with both original frits in place; this strategy also corrected the problem (Figure 3). Could you comment on these results and suggest how to keep this problem from happening again?

JWD: I think the primary problem was the blocked inlet filter. Low-pressure mixing systems work by opening one proportioning valve for a short time, closing it, and opening the second valve. For example, if the total valve cycle were 100 ms and a 50% B mixture was requested, the A valve would open for 50 ms, and then the B valve would open for 50 ms. The pump would provide a steady 2-mL/min flow throughout. During each cycle, 100 μL of each solvent would be drawn into the mixer. If the inlet line for solvent A were restricted, it would allow less than 100 μL to enter. The pump would try to remove 100 μL from the mixer during the A cycle, generating a slight vacuum in the mixer. When the B valve opened, a surge of the B solvent would relieve this vacuum, resulting in the addition of more than 100 μL of the B solvent during the cycle. The net result would be a mobile phase artificially enriched with B solvent.

A mobile phase rich in B solvent would elute analytes earlier, which is consistent with the problem observed in Figure 1 and corrected in Figure 2. After removal of the A inlet frit, a partial (but less severe) blockage of the B frit would have the reciprocal effect. In both cases, I would expect the precision of the mixture to suffer because the solvent would surge into the pump rather than flowing smoothly, as expected. All of these causes and effects are consistent with your observations.

Further inspection of Figures 2 and 3 supports your guess that the B frit may also be partially blocked. The second analyte is eluted under isocratic conditions during the hold at the beginning of the run (100% A in Figure 3). Because only the A reservoir was being used, we know that analytes 1 and 2 exhibit the true retention times for a 62% methanol mixture. In Figure 2, the first two analytes are eluted noticeably later than they are in Figure 3. This observation suggests that the mobile phase is rich in water, which would be expected if the B frit was partially blocked.

Premixing the mobile phase seems to fix the problem because it allows all the analytes to be retained significantly during the isocratic hold. My guess is that if you removed both reservoir frits, the retention times of the last two analytes would be somewhat shorter. The relative starvation of solvent A in Figure 2 also occurs in Figure 3 (the frits are on both lines), and solvent B is artificially enriching the mobile phase during the gradient.

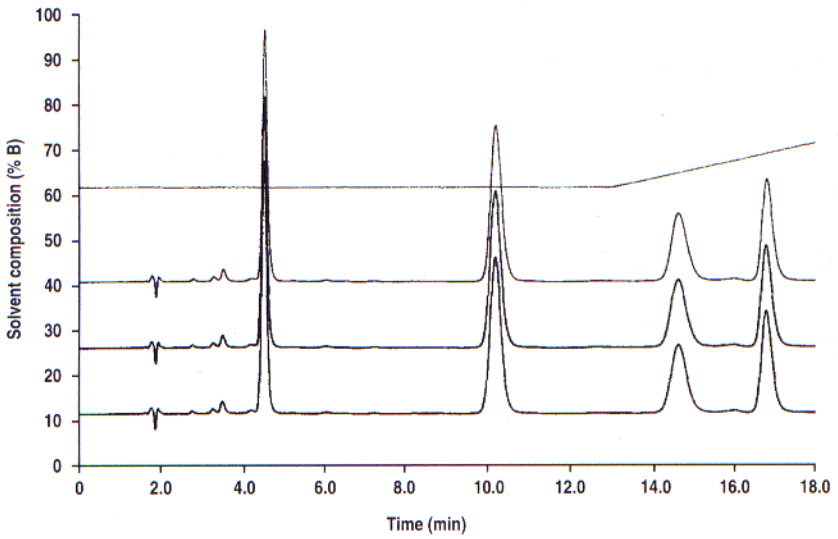

FIGURE 3: Three consecutive runs using premixed mobile phase with original inlet line frits installed. Conditions are described in the text.

I think you will get satisfactory results with your original mobile-phase conditions if you replace both frits with new ones. Premixing the mobile phase is necessary only if you see run-to-run retention variations.

An ounce of prevention: Several precautions can minimize future occurrences of this problem. First, replace the aqueous mobile-phase component more regularly. A pH 7.5 weak solution of diethylamine is a very attractive medium for microbial growth. Daily replacement of the A solvent should reduce the number of problems from this source.

You should also implement a program for regular reservoir-frit replacement. My experience is that replacing these frits twice a year is reasonable, causing little inconvenience and contributing insignificantly to the overall expense of LC operation. If you continue to observe problems, monthly replacement may be necessary. After all, the time you spent chasing this problem cost a lot more than a couple of reservoir inlet-line frits.

Another way to reduce microbial growth problems, especially if making fresh mobile phase each day is impractical, is to premix at least 20% organic in the mobile phase. If you decide to premix, I think preparing a 62% methanol mixture would make sense — it would improve the precision and simultaneously reduce the potential for microbial growth.

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