LC TROUBLESHOOTING

Retention-Time Drift — A Case Study

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Problems like these could happen to anyone. Be alert so they don't happen to you.

The case study presented in this month's column illustrates a number of problems that can arise during method development in liquid chromatography (LC). You are unlikely to encounter all of these problems when developing a single method, but the technician in this case did. One can blame the technician's inexperience or the lack of adequate supervision by an experienced chromatographer, but the fact remains that problems such as these can and do occur with surprising regularity in too many laboratories.

A laboratory needed a method for routinely analyzing several pharmaceutical antidepressant compounds. Because the technician responsible for the assay lacked experience with these compounds, she decided to use a method that had been developed by another laboratory. She searched catalogs and applications notes from LC column manufacturers — the most convenient sources of separation information. The technician located a separation of five compounds of interest with retention times < 10 min. The system used a cyano bonded-phase column and a 25:50:25 (v/v/v) 20 mM phosphate buffer (pH 7)–acetonitrile–methanol mobile phase. Because a cyano column was available in the laboratory, she tried this method.

PROBLEMS BEGIN

The technician had taken an introductory LC class and knew that ternary mobile phases often are unnecessary, so she decided to use only methanol for the organic component to determine whether she could obtain a satisfactory separation. She prepared a buffer of 20 mM dibasic sodium phosphate and, after some experimentation, found that all five peaks could be separated using 80:20 (v/v) methanol–buffer. Although the peaks were well separated, they all tailed unacceptably, so the technician added triethylamine to reduce peak tailing. When she added 30 mM triethylamine to the buffer and readjusted the pH to 7 by adding 20 mM phosphoric acid, the resulting separation was satisfactory. The system pressure was ~1800 psi.

At this point, she examined the separation's reproducibility. She observed consistent retention-time drift; the peaks were eluted later with each successive injection. Because the buffer might not have been strong enough, she prepared a new buffer of 100 mM dibasic sodium phosphate with 100 mM triethylamine (pH 7). Table I shows the results for several of these runs performed on day 2. The first five injections indicated a regular drift of ~0.03 min/run; the remaining injections were unevenly spaced in time, and the drift increased between runs. The technician flushed the system with water and methanol and left it for the night.

Drift continues: The system was restarted on day 3, and the retention times were similar to those recorded at the beginning of day 2, before drifting occurred. The system appeared to have reset itself overnight. However, the first three injections indicated that the drift problem had not disappeared. Thinking that the column might require conditioning by the sample to stabilize retention times, the technician injected several 1-mg samples to accelerate the conditioning. After the baseline stabilized, she continued to inject sample, but the retention-time drift persisted (day 3, runs 4–6) and in some cases worsened. Next, she flushed the column with methanol to restore the original retention conditions. Following this flush, the retention times increased dramatically and continued to increase in larger and larger steps (runs 7–9). Finally, she flushed the column with water, and the retention times dropped back to where they had started on day 3 (runs 10–12). She flushed the system with water and then methanol and left it for the night.

The technician observed the original retention and drift again on day 4 (runs 1–3). At this point, she suspected that something in the mobile phase, not in the sample, was the problem. To amplify the problem, she flushed the column with mobile phase for 60 min (60 mL). A step increase and then a larger drift occurred (day 4, runs 4–7), just as had happened after flushing with methanol on day 3 (runs 7–9). This pattern supported the hypothesis that something in the mobile phase was causing the problem. To isolate the source of the problem, the technician prepared fresh buffer and flushed the column with water before continuing. The next few runs (day 4, runs 8–14) showed the now-familiar sequence: the retention times dropped and then increased regularly after flushing with water. Finally, she flushed the column with mobile phase for 1 h, and the jump in retention times observed earlier recurred (compare day 4, runs 3 and 4 and runs 14 and 15). The technician again flushed the system with water and methanol and left it for the night.

Then, pressure problems: On the following day, she prepared mobile phase without triethylamine to see whether the triethylamine was the source of the problem. With this mobile phase, the pump lost its prime, and the remainder of the day was spent repairing it. The same symptoms were observed the next day as well.

At this point, the technician hypothesized that buffer precipitation in the pump was causing the loss of prime. Experienced workers, who had no previous interest in the problem, offered many suggestions, including using another buffer salt, changing the pH, and changing the buffer concentration. Over the next several days, the technician implemented

TABLE I: Retention-Time Data

Run	t_R (min)* Day 2	Day 3	Day 4	Day 12
1	6.26	6.48	6.55	6.11
2	6.29	6.50	6.58	6.10
3	6.32	6.51†	6.59†	6.10
4	6.36	6.58	6.70	6.10
5	6.41†	6.63	6.73	6.10
6	7.79	6.68†	6.79	6.10
7	9.02	6.98	6.86†	6.10
8	11.28	7.43	6.51	6.10
9	—	9.31†	6.58	6.10
10	—	6.65	6.60	6.10
11	—	6.65	6.62	6.10
12	—	6.65	6.65	—
13	—	—	6.69	—
14	—	—	6.74†	—
15	—	—	6.87	—

* Last band.
† A change in operating conditions was made following this run; see text for details.

many of these suggestions, and, as is often the case in such situations, more than one variable was changed at a time and not all of the changes were recorded.

After trying several buffer salt–pH–organic solvent combinations, the technician obtained a satisfactory and reproducible separation (day 12). A final mobile phase of 65:35 (v/v) methanol–25 mM monobasic potassium phosphate (pH 2.9) was selected for routine use.

WHAT WENT WRONG?

Let's review the problems encountered in the development of this assay and see what we can learn from them. First of all, an effort should be made to regularly record the system's operating parameters. In this case, the system pressure was an important symptom of the problem, yet the operator was unsure whether the pressure was stable when she initially observed the retention-time drift. She first realized that pressure problems were occurring when the pump lost its prime on the fifth day. Some LC systems automatically note the pressure on each analysis report; if yours does not, check the system pressure several times a day for abnormalities and record it at least once a day.

> ***As is often the case, more than one variable was changed at a time, and not all changes were recorded.***

The first operator-induced problem occurred on day 2, when the technician increased the buffer concentration. Generally, the buffer concentration should be >20 mM, but concentrations > 50 mM seldom are an advantage in reversed-phase LC. (Higher buffer concentrations can be used in hydrophobic-interaction and ion-exchange LC.) If you suspect that the buffer concentration is too low, increase it by a factor of two; you should observe a change in the separation. Using buffer concentrations > 50 mM, especially those with high organic solvent concentrations, greatly increases the danger of buffer precipitation. Furthermore, LC systems that use low-pressure mixing (as was used here) are likely to have buffer precipitation problems because discrete aliquots of pure solvent or buffer are dispensed into the mixer. These conditions are more likely to cause localized precipitation problems than when using high-pressure mixing, in which both mobile phase components are added at a constant rate. However, both systems can provide satisfactory results when the appropriate buffer concentrations are used.

Although buffer precipitation was not observed initially (perhaps because the pressure was not monitored), a higher buffer concentration did not noticeably improve the method's performance. When you test potentially unstable conditions and find no improvement, restore the original conditions. This was mistake number two.

The sample-loading study on day 3 was a good idea; occasionally, a column has to be primed with several sample injections before the separation stabilizes. When column priming is required, the sample molecules are thought to be saturating some strongly retentive sites on the column; once these sites are filled, the retention stabilizes. For the first few injections, this phenomenon is usually accompanied by increasing peak heights and areas, but no data are available on these parameters in the present study.

The results from flushing with methanol and water on day 3 should have raised a red flag, but the supervisor didn't ask the right questions. When the technician reported that the column had been flushed with strong solvent, the supervisor assumed that this meant methanol. The technician, however, who was a bit confused about strong and weak solvents, thought that any pure solvent was a strong solvent. In reversed-phase LC, of course, the organic component of the mobile phase is the strong solvent and the aqueous component is the weak solvent. In this case, the fact that a water flush caused a step reduction in retention times (for example, day 3, run 10) and a mobile phase or methanol flush increased retention times roughly in proportion to volume (for example, day 3, run 7) indicates that a water-soluble substance was accumulating in the system. This evidence, and the use of a 100 mM buffer, should have made buffer precipitation the obvious suspect, but the likelihood of buffer precipitation wasn't realized until day 5.

The final mistake is one that all of us are guilty of more often than we like to admit. After having received numerous suggestions from other chromatographers, the technician changed too many things at once and was unable to pinpoint which change was primarily responsible for fixing the problem. One can attribute the results to the changes made, but such an explanation lacks specific proof. For example, for the basic compounds used here, a lower pH (2.9 vs. 7.0) should reduce band tailing. This effect could explain how a satisfactory separation resulted at pH 2.9 without the addition of triethylamine. On the other hand, we have no proof that the triethylamine or sodium salt did not contribute to the problem. Changing from the sodium salt to the potassium salt may or may not have made any difference; most workers use potassium salts when preparing LC buffers.

The most likely cause of all of the observed problems is the precipitation of buffer salts either in the low-pressure mixer or in the pump-inlet check valve. Such precipitation could have gradually starved the pump and subsequently reduced the flow rate, thus causing longer retention times. Washing the pump with water quickly dissolved the precipitate and restored the original conditions. Washing it with mobile phase or methanol merely exacerbated the problem by causing further precipitation of the salts.

Another cause of retention-time drift in low-pressure mixing systems — partial blockage of an inlet-line frit — was not a factor in the present example. When an inlet-line frit is blocked, retention times tend to drift in one direction and return to normal only after the frit is replaced. Frit blockage restricts the delivery of one solvent, resulting in the formation of a partial vacuum in the mixer when that solvent is metered in. When the proportioning valve for the second solvent is opened, the second solvent rushes in and fills the void left by the first. As a result, the mobile phase contains a higher proportion of the second solvent than desired. If the frit remains blocked, as is the case when aged buffers support microbial growth, the solvent proportions continue to change, resulting in retention-time drift.

CONCLUSION

This case study provides an important reminder to keep careful records during method development and to get a different perspective on a problem by asking for the advice of others as soon as an obvious solution doesn't come to mind. Furthermore, it encourages us to keep track of system pressure on a regular basis and to avoid using unreasonably high buffer concentrations.

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Bulletins

Biotechnology market expected to exceed $1 billion. A new report released by Market Intelligence Research Corporation (MIRC) projects world revenues of more than $1 billion for the biotechnology instrumentation market in 1992. According to "World Biotechnology Instrumentation and Software Markets," one reason for this expected growth is the increase in sales of polymerase chain reaction instrumentation; the report forecasts a revenue increase of nearly 21% by 1996. The analytical instrument market, however, is expected to show a decline from 71% to 57% of the world market. For more information on the 292-page report, contact MIRC USA, 2525 Charleston Road, Mountain View, CA 94043, tel. (415) 961-9000.

PerSeptive Biosystems expands facilities. PerSeptive Biosystems (Cambridge, Massachusetts) has expanded its manufacturing facilities to enable increased production of the company's Perfusion Chromatography media, designed for use in high-speed protein and peptide separations.