Troubleshooting

The Problem of Late Elution — A Case Study

Roy Lewis and John W. Dolan

When is the run over?

Sometimes it is hard to know when the last peak has been eluted from a liquid chromatography (LC) column. If the method is developed by end users, it is likely they will have used a gradient to a strong solvent at some time during development. Strong-solvent conditions are expected to provide information about the presence of strongly retained solutes in the sample. These data can be used to build conditions into a method that minimizes problems related to late elution. However, if a method is developed in one laboratory and transferred to a second laboratory for routine use, users may be in for some surprises. This month's installment of "LC Troubleshooting" uses a case study that illustrates some potential problems that may arise during method transfer.

The Separation

A method to quantify the presence of three known impurities within in-process samples was developed and validated in one laboratory. The method then was transferred to a second laboratory for routine use. The transfer protocol called for a simple duplication of results for the same set of samples run in both laboratories.

The chromatographic conditions comprised a 250 mm × 4.6 mm, 5-µm d_p C18 column, an isocratic mobile phase of 35:65 acetonitrile–buffer (25 mM phosphate–triethylamine, pH 3), a flow rate of 1.5 mL/min, detection at 214 nm, and an injection volume of 10 µL.

The initial injection of a mixture of the impurity standards provided results similar to those found in the originating laboratory. As Figure 1 shows, the impurity peaks of interest at 5.5 and 6.5 min were well-shaped with a U.S. Pharmacopeia (USP) tailing factor of less than 2, whereas the peak at 14.8 min had marginally acceptable tailing with a USP tailing factor of approximately 2.1.

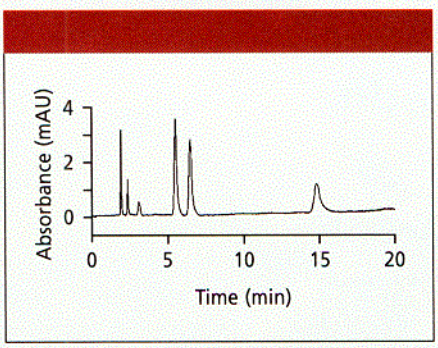

Figure 1: Chromatogram showing desirable characteristics for peaks of interest at 5.5, 6.5, and 14.8 min.

The Problem

Although the first injection or two looked comparable to the results from the original laboratory, subsequent injections showed variable peak shape. Figure 2 shows two examples. The peak shape was not reproducible from run to run: sometimes it was satisfactory and other times it exhibited fat, tailing, or doubling peaks, such as those in Figure 2. In addition to the poor peak shape, the peak area precision dropped so that the relative standard deviation exceeded the 5% method limit. In some chromatograms, increased baseline noise was present.

One likely cause for tailing or peak doubling is a blocked frit or void at the column head.

Check the Obvious

When peak distortions such as tailing or peak doubling occur for all peaks in a chromatogram, one likely cause is a blocked frit or void at the column head. The distortion of the first three peaks in the chromatograms of Figure 2 is not obvious, but the

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narrow peak widths may be hiding any problems. The first and easiest way to determine if the column was the source of the problem was to replace the column with a new one. The resulting chromatograms were similar to those of Figure 2, so the column was not the culprit.

We studied the issue of poor precision by performing an autosampler precision test. Replicate injections of a test mix under standard test conditions resulted in relative standard deviations of less than 1%, which is well within the performance specifications. This test showed that the area precision problem was unrelated to the autosampler performance. Next, we carefully cleaned the autosampler to ensure that the extraneous baseline noise and peak doubling was the result of carryover or contamination by the autosampler.

Some of the baseline noise appeared to be in a regular pattern, suggestive of a malfunctioning pump, an air bubble in the detector, or a failing detector lamp. Figure 2a from approximately 10 min to the end of the run shows this pattern. A pump delivery problem, caused by either an air bubble in the pump or a leak, should reduce the flow rate, thus increasing the retention time of peaks. Furthermore, flow-rate disturbances rarely are reproducible, so we would expect that the flow rate would change from run to run. Because the retention volume in isocratic separation should be constant, any run-to-run fluctuation in flow rate should decrease the retention-time precision. The retention times of the peaks of interest were very consistent, with a variation of only ±0.01 min for the 14.8-min peak in five replicate injections. This consistency indicated that the flow rate precision was quite good and gave the pump a clean bill of health. Neither increased mobile-phase degassing nor replacement of the detector lamp resulted in any dramatic improvement in the baseline quality, thus removing air bubbles and detector problems as possible primary suspects.

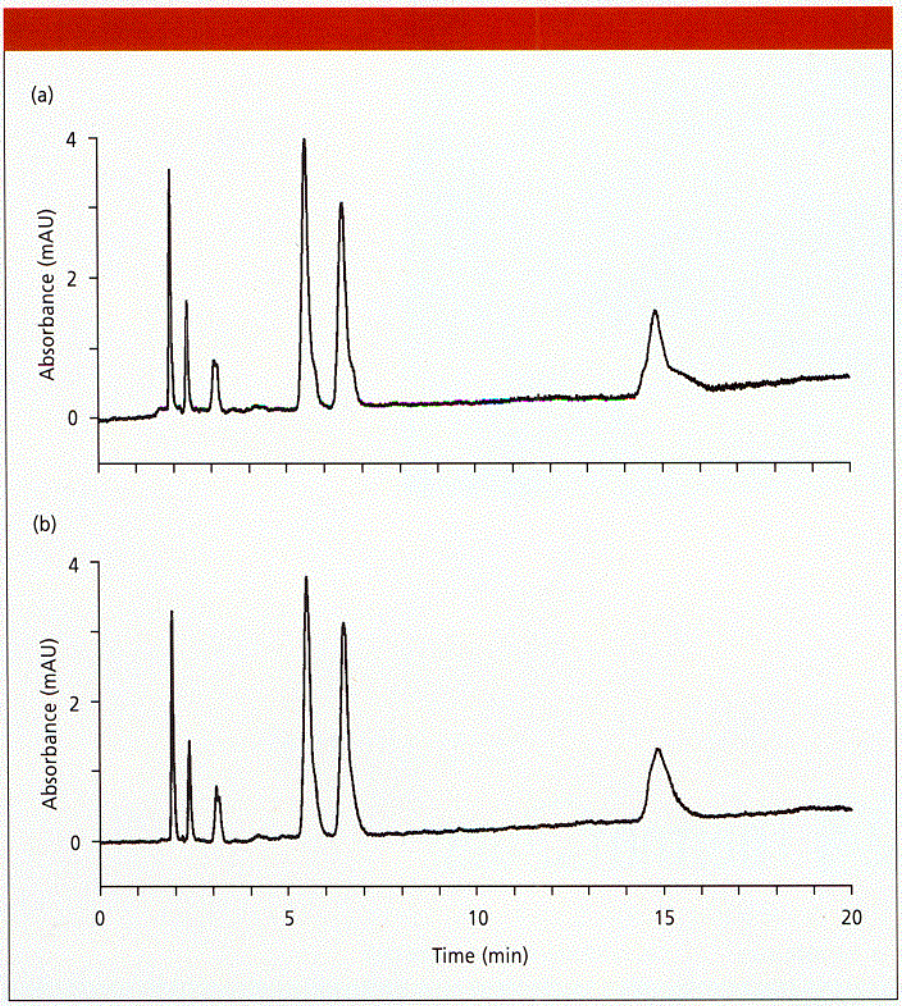

Figure 2: Two examples of chromatograms showing distorted peaks under the same conditions as Figure 1.

What About the Baseline?

In addition to the undesirable baseline noise, our close examination of the chromatograms in Figures 1 and 2 revealed a gradual rise in the baseline throughout each run. This type of baseline drift is common with gradient elution separations, but isocratic separations should produce nondrifting baselines if the column temperature is constant, as it was in this case. To study the baseline drift, we extended the run time from 20 min, as in Figures 1 and 2, to 180 min. Figure 3 shows three 60-min segments of the baseline. The absolute retention times of the three peaks of interest are shorter than for Figures 1 and 2 because the flow rate was increased and a different column was used. However, we could make two significant observations from the run of Figure 3.

The presence of strongly retained materials in real-world samples can result in peaks that are eluted long after the normal run.

First, the baseline continues to rise throughout the entire run. This increase indicates that something is bleeding off the column. Second, several distinct broad peaks are present, for example, near 70 and 150 min. Baseline rise is normal in gradient elution because the B solvent (for example, acetonitrile or methanol) generally has a stronger UV absorbance than the A solvent (for example, water or buffer). Because the solvent composition doesn't change in an isocratic separation, this kind of drift is unexpected. If a UV-absorbing contaminant were present in an isocratic mobile phase, we still would expect to see a constant baseline level. The baseline might be higher than true zero but would be unapparent because of detector autozeroing at the beginning of each run. Therefore, dirty mobile phase can be eliminated as a possible problem source.

A more likely source of the rising baseline and the extra peaks is the sample itself. The sample was an in-process sample that was injected directly onto the LC column. Although the sample appeared to be clear, it could contain any number of compounds

that absorb in the low-UV region. The baseline rise does not negatively affect the separation, but the extraneous peaks could be coeluted with sample components in later runs. This peak overlap could appear as distorted or tailing peaks, and we would expect it to increase the peak area variance. Because late-eluted peaks from one run might appear several runs later, the presence of interfering peaks could be variable. For example, if we used a 20-min cycle time, a peak normally eluted at 30 min in run 1 would appear in the next run at 10 min, but a peak at 50 min wouldn't appear until the third run.

When strongly retained peaks are present in a sample, the problem can be addressed in one of two ways. First, analysts can change the sample pretreatment steps to avoid injecting the problem compounds. For materials that are retained strongly under reversed-phase conditions, solid-phase or liquid–liquid extraction could provide easy removal. However, using sample cleanup with an in-process impurity assay is risky, because a worker could unintentionally remove impurities that should be quantified.

Second, users can flush strongly retained materials from the column before they are

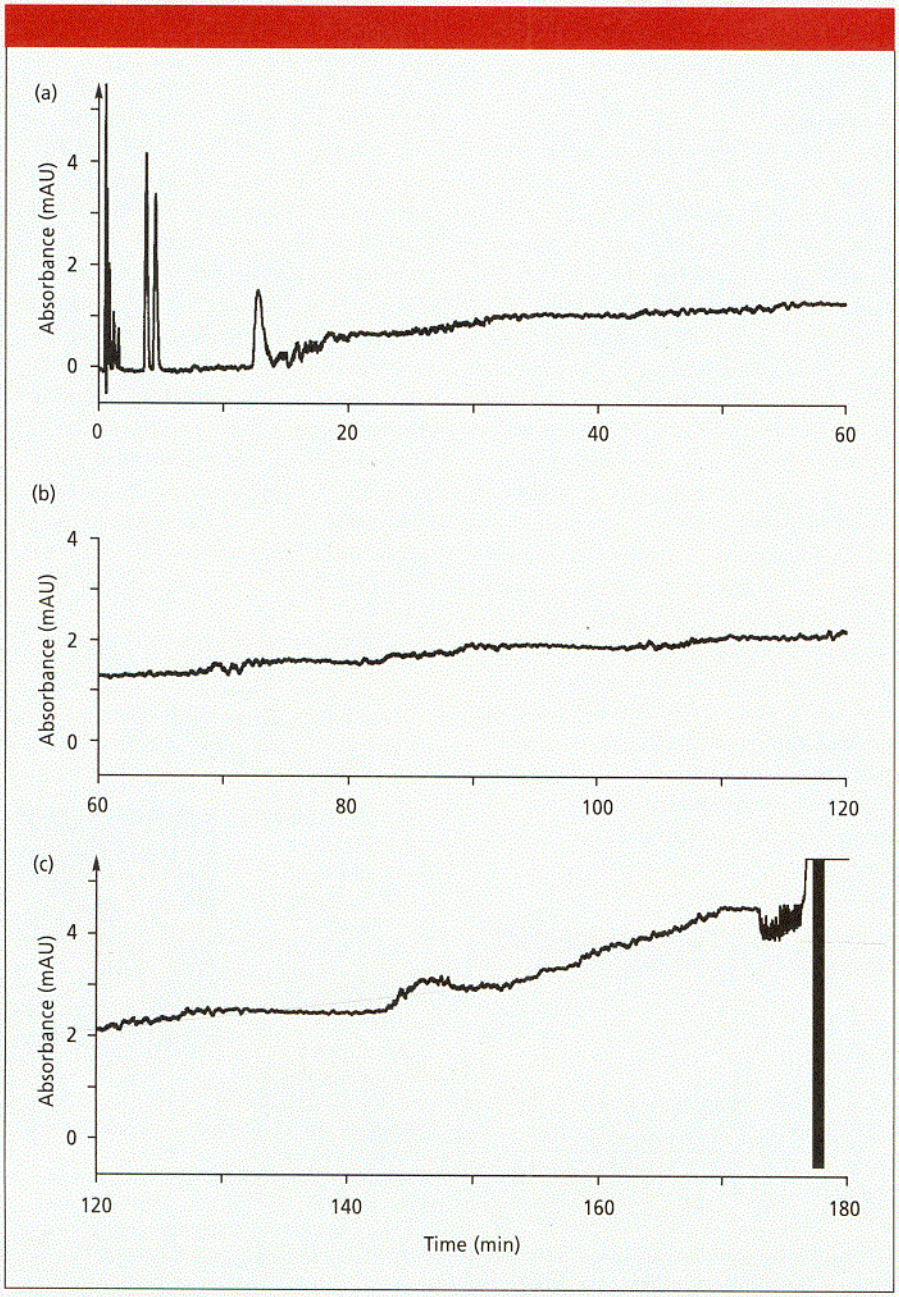

Figure 3: Sections of a chromatogram from an extended isocratic run. Shown are (a) 0–60 min, (b) 60–120 min, and (c) 120–180 min sections of the run. Conditions were similar to those of Figures 1 and 2, but with an increased flow rate and a different column.

eluted and cause problems. In the present example, we used a strong solvent flush to strip these unwanted peaks from the column. As soon as the peaks of interest were eluted, we changed the mobile phase from 35% acetonitrile–buffer to 95% acetonitrile–buffer. After 10 min, we stepped the mobile phase back to the 35% acetonitrile run conditions and re-equilibrated the column. Figure 4 shows the resulting chromatogram. Compare the peak shape for the three analytes in Figure 4 with those in Figure 2 — the retention times are different because of flow rate and column differences, and the improvement is dramatic. The long-term baseline noise improvement is marginal, but the drift seems to be reduced when we compare the baseline of Figure 4 with that of Figure 3.

Summary

The presence of strongly retained materials in real-world samples can result in peaks that are eluted long after the normal run time is over. These peaks can cause three kinds of problems in later runs. If the peaks are large and well-behaved, they can show up as a real peak in a subsequent run. In isocratic separation, these peaks can be identified easily because they will be significantly broader than neighboring peaks. Intermediate or small peaks, such as those in our case study, can be eluted in later chromatograms, but they are small and broad enough that they do not appear as traditional Gaussian peaks. Instead, they may be eluted under a peak of interest and cause distortion, as in our present case, or as broad peaks outside the region of interest. Finally, peaks eluted late in a run can be small enough or so strongly retained that they appear only as a baseline hump. The result is a baseline with irregular humps and bumps that make it difficult to quantify small peaks of interest.

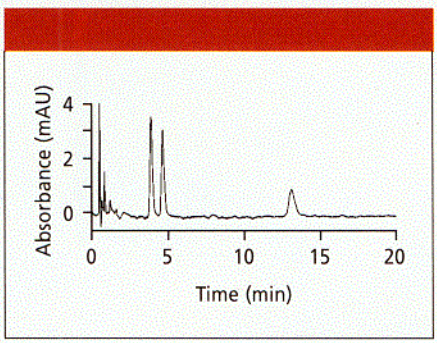

Figure 4: Typical chromatogram obtained after incorporation of a strong-solvent flush in the method. Conditions similar to those of Figure 3.

In each case, flushing the column with a strong solvent should remove the strongly retained materials. Sometimes it may be necessary to flush the column after each run; at other times, it may be sufficient to flush the column once a day. In any event, it is a good idea to flush the column with a strong solvent at the end of each run sequence so that any strongly retained materials are flushed from the column before its next use.

Another problem illustrated in our case study is the common situation in which a strong cause-and-effect relationship is not clear. The peaks in the well-behaved runs of Figures 1 and 4 do not behave ideally — they exhibit significant band tailing we would like to eliminate. Where do the extra peaks in the 15–20 min region of Figure 3a come from? They were not present in any of the other runs. What happened in the 170–180 min region of Figure 3c? Was it an air bubble or something reproducible? Running two or three more 3-h runs to confirm reproducibility doesn't seem like a wise use of time. How about the choppy noise in the 10–20 min region of Figure 2a? No intentional action seemed to remove it, yet the problem disappears later. The bottom line on this problem is that a strong-solvent flush allowed the method to be used for its intended purpose. The remaining questions have to be filed in the "I dunno" category, without a satisfying answer.

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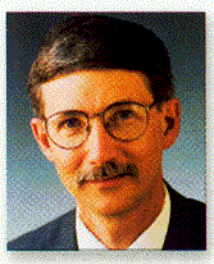

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