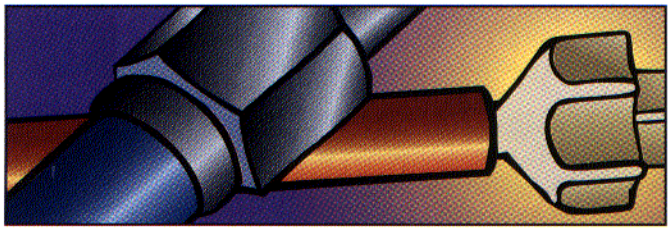
LC Troubleshooting



Water Problems — A Case Study

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Sometimes high performance liquid chromatography grade isn't pure enough.

We recently were developing a stability-indicating assay for a pharmaceutical product. The liquid chromatography (LC) method required that we quantitate all degradant peaks that exceeded 0.1% of the peak area of the parent compound. With UV-absorbance detection at 255 nm, we found that conditions that allowed on-scale detection of the parent peak resulted in 0.1% degradant peak heights of approximately 10^{-4} AU. Such small peaks would not present major problems for a simple sample, but our compound produced nearly 80 degradation peaks, so the chromatogram was very complex. We had developed a gradient method that separated most of the peaks of interest, but a no-injection blank run yielded a chromatogram with at least 10 background peaks that potentially would interfere with the assay. We had to locate the source of the interfering peaks and either remove those peaks or reduce their size to an acceptable level.

THE SETUP

We performed the method on a two-pump, high-pressure mixing LC system with a 2.3-mL dwell volume. Solvent A was 27 mM trifluoroacetic acid (pH 3), and solvent B was high performance liquid chromatography (HPLC)-grade acetonitrile. Both solvents were degassed by helium sparging except where noted. We used two brands of columns (A and B) in the current examples, both 15 cm × 4.6 mm C18 columns. The flow rate was 1.5 mL/min, the column was thermostated at 35 °C, and the UV detector was set to 255 nm. The gradient ran from 0% or 5% B to 83% B with a short isocratic hold at the end. We used a 10-min equilibration time between runs except as noted. The injection size and autosampler details are irrelevant because all of the data reported here are for no-injection runs.

RELATIVE PERFORMANCE

Before trying to isolate a problem, you should determine whether the LC system is working reasonably well. The lower chromatogram in Figure 1 shows a blank gradient for a typical run with column A. The manufacturer's specifications for the detector claim a noise level of 0.5×10^{-5} AU at 250 nm with air in the cell. The noisiest region of the baseline in the 0–4 min region of the chromatogram exhibits peak-to-peak noise of about 1×10^{-5} AU with a gradient running. Thus, the observed performance under real operating conditions is about twice the ideal noise level, so the detector is performing very well.

To get an idea of the magnitude of the background peaks, compare them to a known standard. The acetonitrile manufacturer claims that the solvent will produce no UV-absorbing peak greater than 0.001 AU at 254 nm with a 100% water to 100% acetonitrile gradient. Only the large peak at approximately 12 min exceeds this specification, so the interfering peaks are smaller than those allowed in the HPLC-grade acetonitrile.

WHICH SOLVENT?

To solve this type of problem, it is important to approach it methodically. Two basic troubleshooting rules of thumb are the *Rule of One* and the *Rule of Two*. The Rule of One states that you should change only one variable at a time when searching for the source of a problem. This maxim seems natural enough, but we often ignore it when we are in a hurry. If you change more than one variable at a time, you will not be sure which change corrected the problem. The Rule of Two states that you should make sure that a problem occurs at least twice before you bother trying to solve it. In the same vein, you should duplicate all experiments to be sure that any change occurs consistently. Although only one example of each condition is shown here, all runs were duplicated at least once.

At first, we suspected that the autosampler was contributing the peaks, because a blank injection produced a fairly complex chromatogram. We carefully cleaned the autosampler, replaced the wash solvent, and varied the injection volume of the blank injections. However, these steps did nothing to correct the problem, so we started running no-injection blank gradients. (In programming our LC system, we were able to set the injection volume to −1 and the autosampler skipped the injection, but the rest of the system ran as normal.) The upper chromatogram in Figure 1 shows a typical no-injection gradient.

A simple way to determine if the A or B solvent is the problem source is to increase the equilibration time between runs. When the mobile phase is very weak, sample compounds travel through the column very slowly. If the solvent is weak enough, the compounds exhibit no practical movement. This same process holds for trace impurities in the

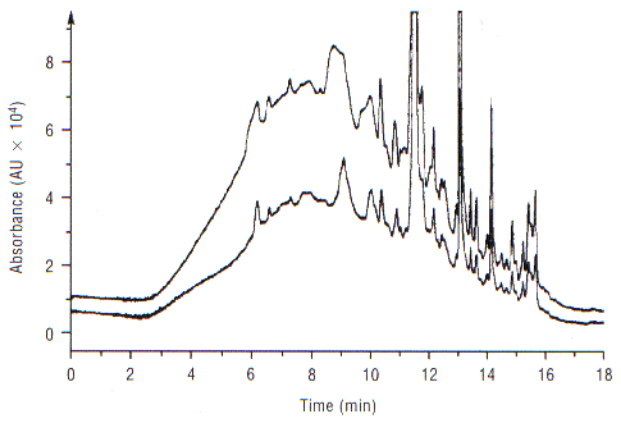

FIGURE 1: Blank gradient runs performed on column A after (lower chromatogram) 10- and (upper chromatogram) 30-min equilibrations. Gradient: 5–83% acetonitrile–trifluoroacetic acid buffer in 13 min, then 83% acetonitrile–trifluoroacetic acid buffer for 5 min.

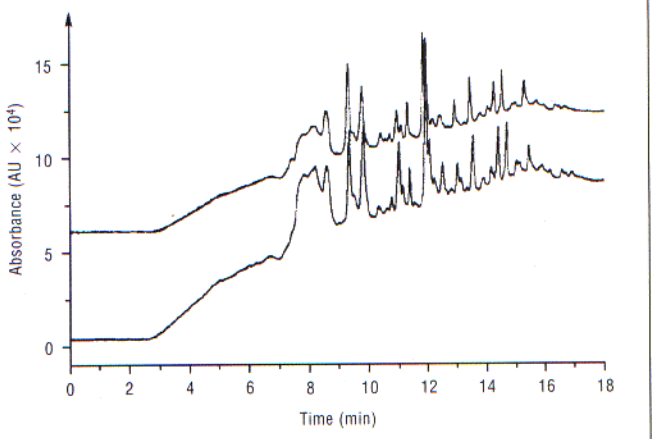

FIGURE 2: Blank gradient runs performed on column B (lower chromatogram) with solvents A and B helium-sparged for 30 min and (upper chromatogram) with fresh solvents and no helium sparging. Gradient: 5–83% acetonitrile–water in 13 min, then 83% acetonitrile–water for 5 min.

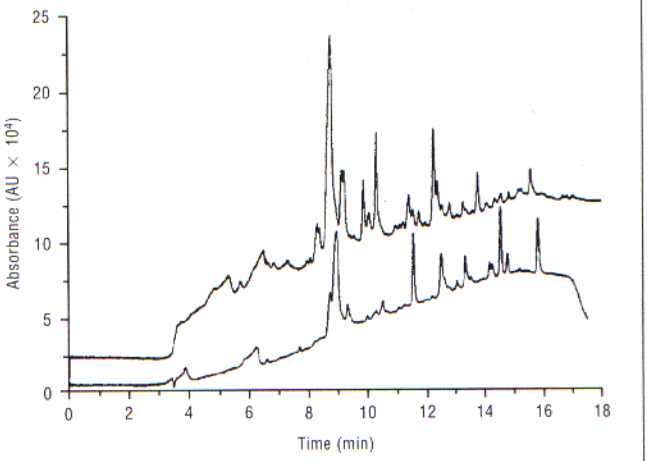

FIGURE 3: Blank gradient runs performed on column B with (lower chromatogram) distilled water purchased from a local grocery store and (upper chromatogram) HPLC-grade water. Gradient: 0–83% acetonitrile–water in 13 min, then 83% acetonitrile–water for 5 min.

mobile phase. When a mobile phase of near 0% B is pumped through the column, impurities in the A solvent tend to build up at the head of the column. This process is called *on-column concentration* and is used to the chromatographer's advantage when large injections are made in a weak solvent. With impurities in the gradient solvents, however, on-column concentration results in buildup of unwanted peaks that appear later in the gradient. As long as the gradient conditions, including equilibration, are kept constant, a fairly constant blank chromatogram will result such as the lower chromatogram in Figure 1. The peaks vary enough from run to run so that they cannot be removed by baseline subtraction, but the peaks are present consistently. By increasing the equilibration time in the A solvent, additional contaminants can build up on the column, resulting in larger peaks as in the upper chromatogram in Figure 1, for which the equilibration time was increased from 10 to 30 min between runs. The peaks are roughly three times as large, thereby confirming our suspicion that solvent A is at fault.

CONTAMINATED REAGENTS?

Now that we knew that the problem was associated with solvent A, we could concentrate on isolating a component of solvent A that contributed the extra peaks. One potential source of contaminants was the buffer salts added to the mobile phase. We were restricted somewhat in the changes we could make, because the current solvents provided the separation we desired — we did not want to start over with a different buffer. However, to check for potential reagent contaminants, we used water instead of buffer for the next set of experiments. The lower chromatogram in Figure 2 shows a blank gradient of 5–83% acetonitrile–water. We performed this separation on column B on a second LC system of the same nominal configuration (same manufacturer and components) and compared the results with the lower chromatogram in Figure 1, which shows the same gradient using trifluoroacetic acid buffer instead of water as the A solvent. The runs exhibit some small differences but for the most part, the chromatograms are much the same, suggesting that the buffer was not the primary source of the extra peaks. We expected some difference in the chromatograms because the separations used different brands of columns.

A DEGASSING PROBLEM?

When helium sparging is used for solvent degassing, analysts must carefully remove the sparging frit from the solvent bottle before turning off the helium flow, or solvent may be drawn back into the helium supply tube. In extreme cases, we have seen mobile phase back up as far as the helium pressure regulator. When this situation occurs, contaminants can migrate from the regulator and ultimately get flushed into the mobile phase. To check for this problem, we compared the baseline produced from blank gradients with and without helium degassing. The upper chromatogram in Figure 2 shows the baseline of a freshly prepared mobile phase (both A and B solvents) with no helium sparging. The lower chromatogram in Figure 2 shows the same gradient after the solvents were degassed for 30 min with helium sparging (this is the normal practice). We observed no significant difference in the number or size of peaks, so we could eliminate the helium sparging (or contamination of the helium lines) as the problem source.

DIRTY GLASSWARE?

Contaminated glassware is a problem that seems to lurk in the shadows to inflict maximum damage on an experiment at the most inopportune time. We hand-wash our glassware using a laboratory detergent, a deionized water rinse, and oven baking. To eliminate contaminated glassware as a potential problem source, we recleaned the glassware, taking extra care to rinse the cleaned glassware with acetonitrile and HPLC-grade water before use. This procedure made no difference in the resulting chromatograms, so we were reassured that our glassware was sufficiently clean.

IS IT THE WATER?

Because of the high potential for chromatographic problems related to contaminated water, we use a commercial system to prepare HPLC-grade water as needed for all our LC applications. Despite this precaution, water impurities can still result, especially if the cartridges are not changed often enough or if a cartridge is placed out of order. For example, if one of the ion-exchange cartridges is placed last (instead of a carbon filter), trace organics from the ion exchanger can contaminate the final water.

Whenever you suspect contaminated water, compare your water with water from another source, such as a bottle of HPLC-grade water that you have purchased from a solvent manufacturer. Another convenient source of water is distilled water available in grocery stores. This water is not as pure as HPLC-grade water, but it is reasonably clean and definitely is from a different source.

For this experiment, we used water as solvent A and acetonitrile as solvent B. Gradients were run from 0% B to 83% B to exaggerate any buildup of contaminants from the water (equilibration at 0% B should accumulate more impurities than at 5% B). The lower chromatogram in Figure 3 shows a gradient formed

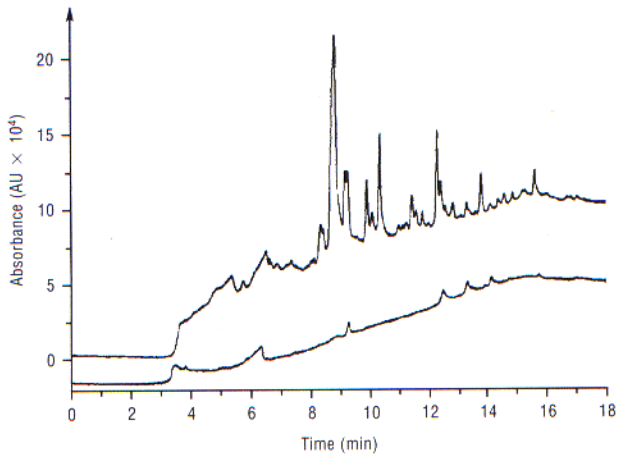

FIGURE 4: Blank gradient runs performed on column B (upper chromatogram) without (same as the upper chromatogram in Figure 3) and (lower chromatogram) with a cleanup column. Gradient: 0–83% acetonitrile–water in 13 min, then 83% acetonitrile–water for 5 min.

using distilled water purchased from a local grocery store, and the upper chromatogram shows a gradient formed using HPLC-grade water under the same conditions. The difference is dramatic; extra peaks appeared in the HPLC-grade water run but not in the run using distilled water, suggesting that contaminants were indeed in our HPLC-grade water.

REMOVING CONTAMINANTS

At this point we determined that the interfering peaks in our blank gradient runs originated in the water used for solvent A. These peaks collected at the head of the column during equilibration and then were eluted as the gradient was run. One way of eliminating such peaks is to trap them on the head of the column and then not elute them. This scheme can be accomplished by placing another C18 column upstream from the mixer to strip organic impurities from the aqueous mobile phase. This technique is limited to high-pressure mixing systems and can significantly increase the system back pressure.

We decided to use a somewhat modified version of this technique. The capacity of a column to remove contaminants under these conditions is proportional to the volume of packing in the column, but the back pressure is proportional to the length of the column. We decided to use a 1 cm × 1 cm column, which has roughly the same capacity as a 5 cm × 4.6 mm column but only 20% of the back pressure. We unpacked an old 25-cm C18 column (same brand as the analytical column), discarding about 2 cm of packing from each end. We slurry-packed this material by hand into a 1 cm × 1 cm guard column (designed for preparative use). The guard column was then washed with approximately 10 column volumes of acetonitrile to remove any strongly retained contaminants. Then the column was placed between the A pump (water or buffer) and the mobile-phase mixer on the high-pressure side of the pump.

The lower chromatogram in Figure 4 shows the resulting blank gradient. Compare this chromatogram with the upper chromatogram in Figure 4, which shows the same blank gradient without the cleanup column in place. This modification significantly improved the baseline, and, although it did not remove all the extra peaks, it allowed us to run our method without interference problems. We run a blank gradient at the beginning of each day to verify that the baseline is sufficiently clean. When the contaminant peaks begin to grow, we can either replace the cleanup column or remove the contaminants from it by flushing with acetonitrile. We have been able to use the cleanup column for approximately three weeks before it required replacement.

CONCLUSIONS

This case study illustrates how a systematic problem isolation scheme can isolate a problem with contaminated water. It should be emphasized that the level of contaminants in the above study was lower than that allowed in commercial solvents. We encountered problems only because our method demanded that we operate our UV-absorbance detector at maximum sensitivity. This problem may have been present for months, but our other methods did not have such demanding requirements.

The use of a cleanup column to strip organics from the mobile phase is not an original idea. In fact, this technique is used in many laboratories because it is so effective at removing trace organics. A short, fat column generally will provide more satisfactory results, because it contributes less to the system back pressure than a longer column of comparable volume. In our case, the level of contaminants and the effectiveness of the column enabled us to use the cleanup column for several weeks without regeneration. In other cases, regeneration may be required more often. One recent article reports the use of a cleanup column in a similar configuration, but it was backflushed to remove contaminants between every injection (1). Finally, this cleanup technique is useful only for high-pressure mixing systems. The use of such a cleanup column in a low-pressure mixing system would require placing it between the reservoir and low-pressure mixer, but the back pressure would be too great to allow solvent to reach the mixer.

REFERENCE

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