LC Troubleshooting

Internal Standard Problems, Buffer Precipitation, and Column Storage

John W. Dolan

This month's "LC Troubleshooting" tackles readers' questions. Column editor John Dolan also provides a list of don'ts for column storage.

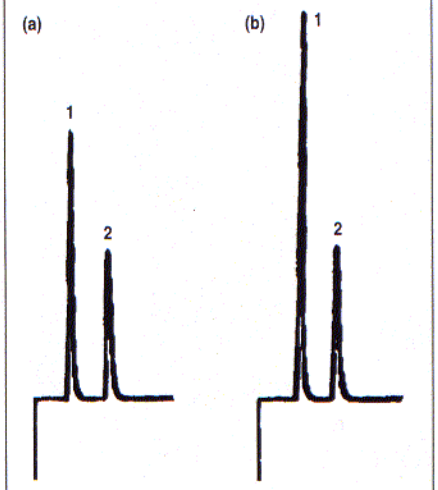

FIGURE 1: Chromatograms showing (a) normal results and (b) an abnormally large internal standard peak. Peaks: 1 = internal standard (t_R = 0.17 min), 2 = analgesic compound (t_R = 0.32 min). Conditions are described in the text.

This month's installment of "LC Troubleshooting" answers questions from three readers. The first letter is about one chromatographer's problems with an internal standard peak that changes in size. The second addresses precipitation that occurs during on-line mixing of organic solvents and buffers. The last question asks how columns should be stored.

INTERNAL STANDARD PROBLEMS

Question: Occasionally, I experience a problem with the internal standard peak during quality-control analyses of an analgesic tablet formulation. Figure 1 illustrates the problem — the internal standard peak (first peak) shows a significant increase in peak height compared with the normal chromatogram. We perform a routine liquid chromatography (LC) analysis and daily run nearly 100 samples, with each sample run twice. The failure rate is approximately 2–3 per 100 samples and does not seem to be associated with the sample or injection number. When I reinject the problem sample, it produces the proper results.

The method uses a 3.3 cm × 4.6 mm, 5-µm d_p C18 column. The mobile phase is 70% acetonitrile–30% 10 mM phosphate buffer. I inject 3 µL of sample using a flow rate of 2.5 mL/min and UV detection at 254 nm. The method is 10 years old and has been quite reliable. The present problem began after a 10-fold increase in our workload.

I unsuccessfully tried to isolate the problem. As I said earlier, the problem does not seem to correlate with the individual sample or the sequence (first or second injection of that sample). I tried the method on two LC systems, and the failure rate seems to be nearly the same. I have also tried replacing the autosampler syringe, changing solvents, and replacing the guard column. One time, I washed the syringe and the internal standard peak increased, but I can't reproduce this result.

The analyte peak always is the correct size, so we are considering a change to an external standard method if we cannot resolve this problem quickly. Do you have any suggestions?

John W. Dolan: Let's start by examining the retention factors of the compounds of interest. The retention factor (k) is calculated as

$$k = (t_R - t_0)/t_0 \quad [1]$$

where t_R and t_0 are the retention time and column dead time, respectively. For the best chromatographic behavior, we would like the retention factor to be between 1 and 20, or better yet, between 2 and 10. When k is less than 1, obtaining reproducible retention times, peak heights, and resolution can be difficult because of injection-related problems and early eluted interferences.

We can estimate the t_0 value using

$$V_m \approx 0.1L \quad [2]$$

where V_m is the column volume in milliliters, and L is the column length in centimeters for a 4.6-mm i.d. column. For other column diameters, we can use the relationship

$$V_m \approx 0.5Ld_c^2 \quad [3]$$

where d_c is the column diameter in centimeters. These two estimates are accurate to roughly 10%, which is good enough for our purposes. After we know the value of V_m, we can convert it to the column dead time using

$$t_0 = V_m/F \quad [4]$$

where F is the flow rate in milliliters per minute.

For the reader's method,

$$V_m = 0.1 \times 3.3 \text{ mL} = 0.33 \text{ mL}$$

$$t_0 = 0.33 \text{ mL}/2.5 \text{ mL/min} = 0.13 \text{ min}$$

So using equations 2 and 4, we determine that t_0 is 0.13 min. Now we can use equation 1 to estimate k for the peaks as 0.3 and 1.4, respectively. This does not include the additional volume contribution by the guard column (the

dimensions were unspecified), so t_R is approximately equal to t_0 for the internal standard. It is not surprising that the reader experienced problems with the internal standard peak. Any change in the injected sample that results in a disturbance at t_0 will affect the peak height (and area) of the internal standard peak. I am surprised, though, that she hadn't encountered this problem before. My guess is that her past success is the result of very clean samples and large analyte concentrations that render the normal disturbance at the column dead time negligible.

Why has the problem occurred only recently? This is hard to determine without further testing. Because reinjection produces a good chromatogram, we can rule out sample contamination. I suspect that the reader may have injected a small air bubble with the sample, which disturbed the internal standard peak. She observed a problem after she washed the syringe — suggesting that the problem may be related to injection, but the results are inconclusive. She should try injecting only air to see if a peak appears at t_0.

How can she work around this problem? Two alternatives are obvious. An easy solution is the one she suggested — using external standard calibration. The analyte's retention is satisfactory and the peak height is stable, so external standardization should work well. Switching to an external standard method should require minimal revalidation.

The alternative to external standardization is to modify the method to increase the retention of the two peaks. The *Rule of Three* provides us with a guideline to determine how large a change in the organic solvent is necessary. The *Rule of Three* states that k changes approximately threefold for each 10% change in organic solvent. I would start by switching to 50% acetonitrile–buffer; this change should increase k by ninefold (3×3). This increase should move the peaks to k values of approximately 2 and 13, or retention to approximately 0.4 and 1.8 min. She may want to make additional adjustments to the mobile phase after obtaining the results of this change. These retention times will yield peaks that behave much better chromatographically, and, by moving the internal standard away from the t_0 region, she should encounter less interference.

I used DryLab software (LC Resources, Walnut Creek, California) and estimated the column pressure at approximately 200 psi under her current conditions. She can regain at least part of the time increase without encountering pressure problems by doubling the flow rate to 5 mL/min. This increased flow rate will provide a method with a subminute run time, acceptable retention factors for all peaks, and higher tolerance for disturbances at the beginning of the chromatogram. Because the sample is simple, her method should require only minimal revalidation.

This example illustrates why we need to examine each method critically. Our tendency is to consider an existing method rugged because it has been in use for a long time. Unstable methods such as the reader's are common — I have seen many routine methods in which the retention is poor, the pH is uncontrolled, or the wrong buffer is used. Although I stress the adage "better is the enemy of good enough," it is important that chromatographers spend enough time to develop methods that are unaffected by the expected variations in conditions. If retention is insufficient, problems are likely, as this reader discovered. The widespread nature of retention problems caused some experts to estimate that more than half of the chromatographic problems would be solved if the methods were adjusted so that $1 < k < 20$.

BUFFER PRECIPITATION

Q: When mixing organic solvents and buffers on-line, I sometimes encounter precipitation problems. After precipitates form, they are difficult to remove, so I would like to avoid these conditions if possible. In my applications, an acetonitrile–buffer mixture seems to be especially problematic with low-pressure mixing. Can I can apply any tricks to minimize precipitation problems?

JWD: In my experience, the best way to avoid buffer precipitation is to minimize the concentration gradient between the two solvents being mixed. The easiest way to do this is to premix the solvents to some degree. For example, if you are using intermediate concentrations of organic solvent, premix 10% organic solvent in the buffer and 10% buffer in the organic solvent. This technique also will improve the mixing characteristics when organic and water are used. A second trick is using the minimum concentration of buffer that is acceptable for your method. For most reversed-phase methods, a final buffer concentration of approximately 25 mM usually is sufficient. Higher buffer concentrations may yield little, if any, chromatographic improvement but will increase the possibility of precipitation. Acetonitrile is more problematic than methanol because the solubility of buffers generally is lower in acetonitrile. Obviously, you need to check the influence of buffer concentration on your separation before selecting the final recipe. An easy way to check for potential buffer precipitation is to add the organic solvent drop-wise to a vial of the proposed buffer. Similarly, try the same test by adding buffer to the pure organic solvent. If you observe visible cloudiness or precipitation, you have a potential solubility problem — correct this problem before proceeding.

If you do encounter a precipitation problem in your LC system, it may be difficult to remove the precipitates. For some reason, precipitated buffer is very difficult to resolubilize after it is in the LC system. You may be able to disassemble the system and sonicate the blocked parts in warm water to remove the deposits. Replacement usually is more convenient for inexpensive parts such as connective tubing. My experience is that after buffer precipitates in the column, you may as well replace the column — buffer precipitated in the pores of the packing is virtually impossible to dissolve.

COLUMN STORAGE

Q: What conditions should I use to store reversed-phase columns? Some people say to leave them in mobile phase, others tell me to use 100% organic solvent, still others tell me to avoid methanol and acetonitrile. What is the correct technique?

JWD: There is no single way to store columns, but there are some simple guidelines to follow. Consider the conditions under which the column was shipped to you as stable. Most manufacturers ship the column in a testing mobile phase, generally 60–80% methanol–water. If they were concerned about column stability under these conditions — in which a column might remain for a year or more — they wouldn't use them.

I have learned three big *don'ts* for column storage. If you avoid these, you will probably be safe.

- Don't store the column with buffer in it (the exceptions to this rule are some ion-exchange and size-exclusion columns that require buffer storage). Buffers tend to precipitate and act as growth media for microorganisms. Flush the buffer from your LC system and column when you shut down the system.
- Don't store the column in less than 30% organic solvent. If you must use high-water-content storage conditions, add azide (for example, 0.04% sodium azide) to suppress microbial growth.
- Don't store the column without sealing the ends securely. Either leave the column connected to the system or use the end plugs that were supplied with the column. Uncapped columns can lose solvent through evaporation. Although a dry column may not be ruined, it is difficult to remove all the air when the column is used again.

If you remove the column from the LC system for storage, be considerate of the next user and add a label indicating the storage solvent.

The best practice is to store the column in 100% of the organic solvent used in your separation. For example, if you are using 45% acetonitrile–buffer, first flush the column with 5–10 column volumes (10–15 mL for a 15-cm column, see equation 1) of unbuffered mobile phase (45% acetonitrile–water) to remove any buffer. Then flush with 10–20 column volumes of strong solvent (100% acetonitrile) to remove strongly retained materials from the column. Shut off the system for storage. To restart, equilibrate with 10–20 column volumes of mobile phase, and check for complete equilibration by injecting standards until the retention time is constant.

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